AmpFℓSTR® Identifiler™

PCR Amplification Kit

User's Manual

Applied Biosystems

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Contents

1 Introduction

Overview 1-1
- About This Chapter 1-1
- In This Chapter 1-1

Product Overview 1-2
- Purpose 1-2
- Five-Dye DNA Fragment Analysis 1-2
- About the Primers 1-2

Multicomponent Analysis Overview 1-3
- About Multicomponent Analysis 1-3
- How Multicomponent Analysis Works 1-3
- Loci Amplified by the Kit 1-4
- About This User's Manual 1-6

Materials for the Kit 1-7
- Kit Contents 1-7
- Kit Storage and Stability 1-7

Safety 1-8
- Documentation User Attention Words 1-8
- Chemical Hazard Warning 1-8
- Site Preparation and Safety Guide 1-9
- About MSDSs 1-9
- Ordering MSDSs 1-10

2 PCR Amplification

Overview 2-1
- About This Chapter 2-1
- In This Chapter 2-1

PCR Work Areas 2-2
- Setup Work Area 2-2

Amplified DNA Work Area 2-2
PCR Equipment and Materials 2-3
Equipment and Materials Required But Not Supplied. 2-3
Preparing the Reagents 2-5
TE Buffer 2-5
Master Mix 2-5
Preparing the DNA Samples 2-7
DNA Sample Input 2-7
Preparing the Samples 2-7
Performing PCR 2-8
Thermal Cyclers 2-8
Amplifying the DNA 2-8
Amplification Using Bloodstained FTA Cards 2-9

3 Protocol for 310 Genetic Analyzer with Mac OS

Overview 3-1
About This Chapter 3-1
In This Chapter. 3-1
Software Requirements 3-2
Collection Software 3-2
Analysis Software 3-2
Preparing the ABI PRISM 310 Genetic Analyzer 3-3
ABI PRISM 310 Genetic Analyzer 3-3
Setting Up the Instrument 3-4
Setting Up the Run 3-11
Setting the Run Temperature 3-11
Setting the Parameters 3-11
Running Matrix Samples 3-12
Filter Set G5 Module Files 3-14
Overview 3-14
Five-Dye Data Collection 3-15
Overview 3-15
Creating a Five-Dye Sample Sheet and Injection List 3-15
Making a Matrix File 3-18
Matrix Standards 3-18
Making a Matrix File on the ABI PRISM 310. 3-18

Running DNA Samples 3-22
Preparing Samples and AmpFℓSTR Identifiler Allelic Ladder 3-22
Loading Samples. 3-23
Sample Electrophoresis. 3-24
Setting Up Software Parameters 3-26
Setting the Analysis Parameters 3-26
GeneScan Software Results. 3-30
GeneScan Analysis Software 3-30
Information Provided in the Electropherogram and Table 3-30
Standards for Samples 3-32
Off-Scale Data 3-33
Overview. 3-33
Off-Scale Data on the ABI PRISM 310 3-33
Shutting Down the Instrument. 3-34
Ending the Run 3-34
Dedicated Equipment and Supplies. 3-35
Equipment Required 3-35
Reagents Required 3-36
Software and User Documentation Required 3-37

4 Experiments and Results

Overview 4-1
About This Chapter. 4-1
In This Chapter 4-1
Experiments Performed Using the AmpFℓSTR Identifiler PCR Amplification Kit 4-2
Importance of Validation 4-2
Experiments 4-2
Developmental Validation 4-3
8.1.1 Developmental Validation 4-3
PCR Components 4-3
Thermal Cycler Parameters. 4-4
AmpliTaq Gold DNA Polymerase Activation 4-5
PCR Cycle Number 4-5
Accuracy, Precision, and Reproducibility 4-6
8.1.2 Accuracy 4-6

Precision and Size Windows 4-7
Extra Peaks in the Electropherogram 4-18
Causes of Extra Peaks 4-18
Artifacts 4-25
Characterization of Loci 4-27
8.1.2.1 Documentation 4-27
Overview 4-27
Nature of the Polymorphisms 4-27
Inheritance 4-27
Mapping 4-28
Species Specificity 4-29
8.1.2.2 Species Specificity 4-29
Sensitivity 4-31
8.1.2.2 Sensitivity 4-31
Importance of Quantitation 4-31
Stability 4-33
8.1.2.2 Stability 4-33
Overview 4-33
Differential and Preferential Amplification 4-33
Effect of Inhibitors 4-33
Degraded DNA 4-34
Mixture Studies 4-38
8.1.2.2 Mixture Studies 4-38
Mixed Specimen Studies 4-38
Data Interpretation 4-42
Minimum Sample Requirement 4-42
Population Data 4-43
8.1.2.3 Population Data 4-43
8.1.2.3.1 Population Distribution Data 4-43
Overview 4-43
Population Samples Used in These Studies 4-43
AmpFℓSTR Identifiler Kit Allele Frequencies 4-44
Mutation Rate 4-54
Estimating Germline Mutations 4-54
Additional Mutation Studies 4-54
Probability of Identity 4-55
Table of Probability of Identity 4-55

Probability of Paternity Exclusion 4-56
Table of Probability of Paternity Exclusion 4-56

5 *Genotyping for the Macintosh OS*

Overview 5-1
About This Chapter 5-1
In This Chapter 5-1
Using Genotyper Software for Automated Genotyping 5-2
About the Software 5-2
Before Running Genotyper Software 5-2
AmpFℓSTR Identifiler Kit Template 5-3
Using the AmpFℓSTR Identifiler Kit Template File 5-4
Examining Data 5-6
Making Tables 5-7
Understanding the AmpFℓSTR Identifiler Kit Template 5-10
Troubleshooting Automated Genotyping 5-10
About This Kit Template 5-10
Categories 5-10
Offset Categories 5-11
Off-Ladder Alleles and Virtual Alleles 5-12
Kazam Macro 5-13
Kazam (20% Filter) 5-15
Modifying the Template 5-15
Determining Genotypes 5-17
AmpFℓSTR Identifiler Allelic Ladder 5-17
Genotyping Using the AmpFℓSTR Identifiler Allelic Ladder 5-19

A *Technical Support*

Contacting Technical Support A-1
To Contact Technical Support by E-Mail A-1
Hours for Telephone Technical Support A-2
To Contact Technical Support by Telephone or Fax A-2
To Reach Technical Support Through the Internet A-5
To Obtain Documents on Demand A-6
To Obtain Customer Training Information A-6

B Troubleshooting

Overview B-1
About This Appendix B-1
In This Appendix B-1
Troubleshooting B-2

C Laboratory Setup

Overview C-1
About This Appendix C-1
In This Appendix C-1
Lab Design C-2
Sensitivity of PCR C-2

D DNA Extraction Protocols

Overview D-1
About This Appendix D-1
In This Appendix D-1
Introduction D-2
Overview of DNA Sample Types D-2
DNA Extraction Methods D-2
Warnings to Users D-3
Collection and Storage of Samples for DNA Extraction D-3
Proper Collection D-3

E DNA Quantitation

Overview E-1
About This Appendix E-1
In This Appendix E-1
Importance of Quantitation E-2
DNA Quality E-2
Quantitation and PCR Amplification E-2
Using the QuantiBlot Kit E-3
How the Kit Works E-3
Specificity for Primate DNA E-3

Single-Stranded and Degraded DNA E-4
Commonly Asked Questions about the QuantiBlot Kit E-5
How Much DNA? E-5
Multiple Film Exposures E-5
Repeating the Assay E-6
Performing Hybridization and Detection at a Later Time E-6

F References

Index

1 Introduction

Overview

About This Chapter

This chapter describes the contents of the AmpFℓSTR® Identifiler™ PCR Amplification Kit, provides an overview of the kit, and provides safety information.

In This Chapter

This chapter contains the following topics:

Topic	See Page
Overview	1-1
Product Overview	1-2
Multicomponent Analysis Overview	1-3
Materials for the Kit	1-7
Safety	1-8

Product Overview

Purpose

The AmpFℓSTR® Identifiler™ PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 15 tetranucleotide repeat loci and the Amelogenin gender determining marker in a single PCR amplification.

- All thirteen of the required loci for the Combined DNA Index System (CODIS) loci are included in this kit for known-offender databasing in the United States (Budowle *et al.*, 1998a).
- Two additional loci, D2S1338 and D19S433, are included. These loci are consistent with the AmpFℓSTR® SGM Plus™ PCR Amplification Kit.
- The combination of the 15 loci are consistent with several worldwide database recommendations.

Five-Dye DNA Fragment Analysis

The Identifiler kit uses a five-dye fluorescent system for automated DNA fragment analysis. By adding an additional dye, more loci can be multiplexed in a single PCR amplification as compared to the previous 4-dye system. Applied Biosystems PET™ and LIZ™ dyes expand the spectral detection range that can be used on ABI PRISM® genetic analysis instrumentation. Together with 6-FAM™, VIC™, and NED™ dyes, the spectral emission for this five-dye set extends to 660 nm.

About the Primers

The AmpFℓSTR Identifiler kit employs the same primer sequences as used in all previous AmpFℓSTR® kits. A degenerate unlabeled primer for the D8S1179 locus was added to the AmpFℓSTR® Identifiler™ Primer Set in order to address a mutation observed in a population of Chamorros and Filipinos from Guam (Budowle *et al.*,1998b and Budowle e*t al.*, 2000). The addition of the degenerate primer allows for the amplification of those alleles in samples containing this mutation without altering the overall performance of the AmpFℓSTR Identifiler PCR Amplification Kit. The data in this user's manual were generated prior to the addition of the degenerate primer. Data showing equivalence with the degenerate primer will be published.

Non-nucleotide linkers are used in primer synthesis for the following loci: CSF1PO, D2S1338, D13S317, D16S539 and TPOX. For these primers, non-nucleotide linkers are placed between the primer and the fluorescent dye during oligonucleotide synthesis (Grossman *et al.*, 1994 and Baron *et al.*, 1996). Non-nucleotide linkers enable reproducible positioning of the alleles to facilitate inter-locus spacing. By combining the five-dye system with the non-nucleotide linkers for selected loci, the same primer sequences developed for previous AmpFℓSTR kits are used without modification.

Multicomponent Analysis Overview

About Multicomponent Analysis

Multicomponent analysis is the process that separates the five different fluorescent dye colors into distinct spectral components. The four dyes used in the AmpFℓSTR® Identifiler™ PCR Amplification Kit to label samples are 6-FAM™, VIC™, NED™ and PET™ dyes. The fifth dye, LIZ™, is used to label the GeneScan™-500 Size Standard.

How Multicomponent Analysis Works

Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the ABI PRISM® instruments, the fluorescent signals are separated by a diffraction grating according to their wavelengths and projected onto a charge-coupled device (CCD) camera in a predictably spaced pattern. 6-FAM dye emits at the shortest wavelength and is displayed as blue, followed by the VIC dye (green), NED dye (yellow), PET dye (red) and LIZ dye (orange).

Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the dyes (Figure 1-1). The goal of multicomponent analysis is to effectively correct for spectral overlap.

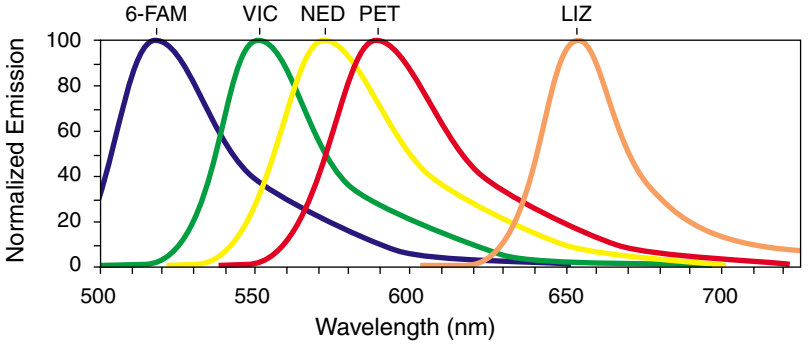


Figure 1-1 Emission spectra of the five dyes used in the AmpFℓSTR Identifiler PCR Amplification Kit

Loci Amplified by the Kit

The table below shows the loci amplified and the corresponding dyes used.

Amplified Loci:

Locus Designation	Chromosome Location	Alleles Included in Identifiler Allelic Ladder	Dye Label	Control DNA 9947A
D8S1179	8	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	6-FAM	13[a]
D21S11	21q11.2-q21	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38		30[b]
D7S820	7q11.21-22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 11
CSF1PO	5q33.3-34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 12
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	VIC	14, 15
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3		8, 9.3
D13S317	13q22-31	8, 9, 10, 11, 12, 13, 14, 15		11[c]
D16S539	16q24-qter	5, 8, 9, 10, 11, 12,13, 14, 15		11, 12
D2S1338	2q35-37.1	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28		19, 23

Amplified Loci: *(continued)*

Locus Designation	Chromosome Location	Alleles Included in Identifiler Allelic Ladder	Dye Label	Control DNA 9947A
D19S433	19q12-13.1	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	NED	14, 15
vWA	12p12-pter	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24		17, 18
TPOX	2p23-2per	6, 7, 8, 9, 10, 11, 12, 13		8[d]
D18S51	18q21.3	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27		15, 19
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	PET	X
D5S818	5q21-31	7, 8, 9, 10, 11, 12, 13, 14, 15, 16		11[e]
FGA	4q28	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2		23, 24

a. For CODIS purposes, profile reported as 13, 13.

b. For CODIS purposes, profile reported as 30, 30.

c. For CODIS purposes, profile reported as 11, 11.

d. For CODIS purposes, profile reported as 8, 8.

e. For CODIS purposes, profile reported as 11, 11.

About This User's Manual

This user's manual provides users of the AmpFℓSTR Identifiler PCR Amplification Kit with protocols and data obtained by Applied Biosystems. Applied Biosystems recommends that users conduct similar experiments to evaluate in their labs the DNA typing system consisting of AmpFℓSTR Identifiler PCR Amplification Kit, reagents, software and ABI PRISM instruments. Furthermore, Applied Biosystems suggests that users apply the standards by the community for which this test will be used to further evaluate this DNA typing system.

This user's manual describes the following:

- Materials and equipment required to use the AmpFℓSTR Identifiler kit
- How to use the kit to amplify DNA samples
- How to perform automated detection
- How to analyze results

Materials for the Kit

Kit Contents

The AmpFℓSTR Identifiler kit contains sufficient quantities of the following reagents and the appropriate licenses to perform 200 25-µL amplifications:

Component	Description	Volume
AmpFℓSTR® PCR Reaction Mix	Two tubes containing $MgCl_2$, deoxynucleotide triphosphates, and bovine serum albumin in buffer with 0.05% sodium azide	1.1 mL/tube
AmpFℓSTR Identifiler Primer Set	One tube containing fluorescently labeled primers and non-labeled primers	1.1 mL
AmpliTaq Gold® DNA Polymerase	Two tubes of enzyme with an activity of 5 U/µL	50 µL/tube
AmpFℓSTR® Control DNA 9947A	One tube containing 0.10 ng/µL human female cell line DNA in 0.05% sodium azide and buffer (refer to pages 1-4 and 1-5 for profile)	0.3 mL
AmpFℓSTR® Identifiler™ Allelic Ladder	One tube of AmpFℓSTR Identifiler Allelic Ladder containing amplified alleles. See the table on pages 1-4 and 1-5 for a list of alleles included in the allelic ladder	50 µL

Kit Storage and Stability

The table below lists the storage temperature for the kit components.

IMPORTANT The fluorescent dyes attached to the primers are light-sensitive. Protect the AmpFℓSTR Identifiler Primer Set from light when not in use. Amplified DNA, AmpFℓSTR® Identifiler™ Allelic Ladder and GeneScan™-500 LIZ™ Size Standard should also be protected from light.

Component	Storage Temperature
AmpFℓSTR PCR Reaction Mix	2 to 8 °C
AmpFℓSTR Identifiler Primer Set	
AmpFℓSTR Control DNA 9947A	
AmpFℓSTR Identifiler Allelic Ladder	
AmpliTaq Gold DNA Polymerase	–15 to –25 °C

Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note Calls attention to useful information.

IMPORTANT Indicates information that is necessary for proper instrument operation.

⚠ CAUTION Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

⚠ WARNING Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.

⚠ DANGER Indicates an imminently hazardous situation which, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

⚠ WARNING CHEMICAL HAZARD. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and could cause injury, illness, or death.

- Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, chemical safety, and waste profiles.

About MSDSs

Some of the chemicals used with this instrument may be listed as hazardous by their manufacturer. When hazards exist, warnings are prominently displayed on the labels of all chemicals.

Chemical manufacturers supply a current MSDS before or with shipments of hazardous chemicals to new customers and with the first shipment of a hazardous chemical after an MSDS update. MSDSs provide you with the safety information you need to store, handle, transport and dispose of the chemicals safely.

We strongly recommend that you replace the appropriate MSDS in your files each time you receive a new MSDS packaged with a hazardous chemical.

⚠ WARNING CHEMICAL HAZARD. Be sure to familiarize yourself with the MSDSs before using reagents or solvents.

Ordering MSDSs

You can order free additional copies of MSDSs for chemicals manufactured or distributed by Applied Biosystems using the contact information below.

<table>
<tr><th>To order MSDSs...</th><th>Then...</th></tr>
<tr><td>Over the Internet</td><td>a. Go to our Web site at www.appliedbiosystems.com/techsupp.
b. Click MSDSs.

<table><tr><th>If you have...</th><th>Then...</th></tr><tr><td>The MSDS document number or the Document on Demand index number</td><td>Enter one of these numbers in the appropriate field on this page</td></tr><tr><td>The product part number</td><td rowspan="2">Select Click Here, then enter the part number or keyword(s) in the field on this page.</td></tr><tr><td>Keyword(s)</td></tr></table>
c. You can open and download a PDF (using Adobe® Acrobat Reader) of the document by selecting it, or you can choose to have the document sent to you by fax or email.</td></tr>
<tr><td>By automated telephone service</td><td>Use “To Obtain Documents on Demand” on page A-6.</td></tr>
<tr><td>By telephone in the United States</td><td>Dial 1-800-327-3002, then press 1.</td></tr>
<tr><td>By telephone from Canada</td><td><table><tr><th>To order in...</th><th>Dial 1-800-668-6913 and...</th></tr><tr><td>English</td><td>Press 1, then 2, then 1 again</td></tr><tr><td>French</td><td>Press 2, then 2, then 1</td></tr></table></td></tr>
<tr><td>By telephone from any other country</td><td>See “To Contact Technical Support by Telephone or Fax” on page A-2.</td></tr>
</table>

For chemicals not manufactured or distributed by Applied Biosystems, call the chemical manufacturer.

2 PCR Amplification

Overview

About This Chapter

This chapter describes how to prepare the master mix for amplifying sample DNA using the AmpFℓSTR® Identifiler™ PCR Amplification Kit, prepare samples and controls, and perform PCR.

In This Chapter

This chapter contains the following topics:

Topic	See Page
Overview	2-1
PCR Work Areas	2-2
PCR Equipment and Materials	2-3
Preparing the Reagents	2-4
Preparing the DNA Samples	2-6
Performing PCR	2-7

PCR Work Areas

Setup Work Area

IMPORTANT These items should never leave the PCR Setup Work Area

- Calculator
- Gloves, disposable
- Marker pen, permanent
- Microcentrifuge
- Microcentrifuge tubes, 1.5-mL, or 2.0-mL, or other appropriate clean tube (for Master Mix preparation)
- Microcentrifuge tube rack
- Pipet tips, sterile, disposable hydrophobic filter-plugged
- Pipettors
- Tube decapper, autoclavable
- Vortex

Amplified DNA Work Area

IMPORTANT The GeneAmp® PCR Systems should be placed in the Amplified DNA Work Area

- GeneAmp® PCR System 9700

-or-

- GeneAmp® PCR System 9600

PCR Equipment and Materials

Equipment and Materials Required But Not Supplied

The tables below list the equipment and materials required in addition to the reagents supplied with the AmpFℓSTR Identifiler kit for PCR amplification.

Required Equipment:

Equipment	Source
GeneAmp® PCR System 9700	Applied Biosystems (P/N N805-0001)
Microcentrifuge	Major laboratory supplier (MLS)
Pipettors	MLS
Vortex	MLS

Required Materials:

Materials	Source
MicroAmp® 96 Well Trays for Tubes with Caps	Applied Biosystems (P/N N801-0541)
MicroAmp Reaction Tubes with Caps, 0.2-mL	Applied Biosystems (P/N N801-0540)
MicroAmp Reaction Tubes (8 tubes/strip)	Applied Biosystems (P/N N801-0580)
MicroAmp Caps (8 caps/strip)	Applied Biosystems (P/N N801-0535)
MicroAmp 96-Well Tray/Retainer Set	Applied Biosystems (P/N 403081)
MicroAmp 96-Well Base	Applied Biosystems (P/N N801-0531)
MicroAmp Optical 96-Well Reaction Plate	Applied Biosystems (P/N N801-0560)
Microcentrifuge tubes, 1.5-mL	MLS
Microcentrifuge tubes, 2.0-mL	MLS
Pipet tips, sterile, disposable hydrophobic filter-plugged	MLS
Tape, labeling	MLS
Tube, 50-mL Falcon	MLS
Tube decapper, autoclavable	MLS
Deionized water, PCR grade	MLS
Tris-HCL, pH 8.0	MLS
0.5-M EDTA	MLS

Preparing the Reagents

TE Buffer

The final concentration of TE buffer is 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0.

To prepare TE buffer:

Step	Action
1	Mix together: ♦ 10 mL of 1 M Tris-HCl, pH 8.0 ♦ 0.2 mL of 0.5 M EDTA ♦ 990 mL glass-distilled or deionized water **⚠ CAUTION** **CHEMICAL HAZARD. EDTA** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. **Note** Adjust the volumes accordingly for specific needs.
2	Aliquot and autoclave the solutions.
3	Store at room temperature.

Master Mix

Prepare the master mix by combining AmpFℓSTR® PCR Reaction Mix, AmpliTaq Gold® DNA Polymerase, and AmpFℓSTR® Identifiler™ Primer Set reagents.

IMPORTANT The fluorescent dyes attached to the primers are light-sensitive. Protect the AmpFℓSTR Identifiler Primer Set from light when not in use. Also protect the AmpFℓSTR Identifiler Allelic Ladder, GeneScan™-500 LIZ™ Size Standard and amplified, fluorescently labeled PCR products from light.

To prepare the master mix:

Step	Action
1	Determine the total number of samples, including controls.
2	**IMPORTANT** Vortex the following reagents for 5 sec: ♦ AmpFℓSTR PCR Reaction Mix ♦ AmpliTaq Gold DNA Polymerase ♦ AmpFℓSTR Identifiler Primer Set **⚠ CAUTION CHEMICAL HAZARD. AmpliTaq Gold DNA Polymerase** may cause eye and skin irritation. It may cause discomfort if swallowed or inhaled. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves.
3	Spin the tubes briefly in a microcentrifuge to remove any liquid from the caps.
4	Select a clean, unused tube for the master mix. **If you are preparing...** / **Then use a...** ≤ 84 samples and controls / 1.5-mL microcentrifuge tube 85–110 samples and controls / 2.0-mL microcentrifuge tube > 110 samples and controls / tube that is appropriate
5	Calculate the required amount of components as shown: **Note** The formulation in the list below provides a slight overfill to allow for volume lost in pipetting. Number of samples X 10.5 µL of AmpFℓSTR PCR Reaction Mix Number of samples X 0.5 µL of AmpliTaq Gold DNA Polymerase Number of samples X 5.5 µL of AmpFℓSTR Identifiler Primer Set
6	Vortex the master mix at medium speed for 5 sec.
7	Dispense 15 µL of master mix per PCR tube.

Preparing the DNA Samples

DNA Sample Input

DNA amplification with the AmpFℓSTR® Identifiler™ kit requires 10 µL of DNA at a recommended concentration of 0.05–0.125 ng/µL.

Preparing the Samples

Note The final volume in each PCR tube is 25 µL.

To prepare the samples:

If you are preparing the	Then...
DNA test sample tube and the sample DNA concentration is ≤ 0.125 ng/µL	Add 10 µL of sample to the PCR tube.
DNA test sample tube and the sample DNA concentration is > 0.125 ng/µL	Dilute a portion of the sample with TE buffer (see page 2-4 for preparation) so that only 0.5–1.25 ng of total DNA is in a volume of 10 µL (final sample concentration is 0.05–0.125 ng/µL).
Positive Control Tube	a. Vortex the AmpFℓSTR® Control DNA 9947A tube (0.10 ng/µL). b. Spin the tube briefly in a microcentrifuge to remove any liquid from the cap. c. Add 10 µL (1 ng) of AmpFℓSTR Control DNA 9947A to the Positive Control Tube.
Negative Control Tube	Add 10 µL of TE buffer (see page 2-4 for preparation) to the labeled Negative Control Tube.

Performing PCR

Thermal Cyclers

Use either of the following thermal cyclers to amplify loci using the AmpFℓSTR Identifiler kit:

- GeneAmp® PCR System 9700
- GeneAmp® PCR System 9600

Amplifying the DNA

To amplify the DNA:

Step	Action
1	Program the thermal cycling conditions. **IMPORTANT** If using the GeneAmp PCR System 9700, select the 9600 Emulation Mode.

Initial Incubation Step	Denature	Anneal	Extend	Final Extension	Final Step
HOLD	CYCLE (28 cycles)			HOLD	HOLD
95 °C 11 min	94 °C 1 min	59 °C 1 min	72 °C 1 min	60 °C 60 min	4–25 °C (forever)

Note If leaving the amplified products in the thermal cycler for more than 18 hr., set the final step to HOLD at 4–25 °C forever. The final step can be held anywhere in this range. Each laboratory should determine the final time and temperature to store PCR products in the thermal cycler.

2	Place the tray in the thermal cycler.
3	Close the heated cover.
4	Start the thermal cycler.
5	Remove the tubes from the instrument block after the PCR is completed.
6	Store the amplified DNA.

If you are storing the DNA...	Then place at...
<2 weeks	2 to 6 °C.
>2 weeks	–15 to –25 °C.

IMPORTANT Protect the amplified products from light.

Amplification Using Bloodstained FTA Cards

FTA™-treated DNA collection cards can be useful for the collection, storage, and processing of biological samples. A small punch of the bloodstained card can be placed directly into an amplification tube, purified, and amplified without transferring the evidence. Our studies have indicated that a 1.2-mm bloodstained punch contains approximately 5–20 ng DNA. Accordingly, an appropriate cycle number for this high quantity of DNA is 25 cycles. It is recommended that each laboratory determine the cycle number based upon individual validation studies.

In the example shown in Figure 2-1, a 1.2-mm punch of a bloodstained FTA card was purified using one wash with FTA Purification Reagent and two washes with 1X TE buffer. After drying at room temperature overnight, the punch was then amplified directly in the MicroAmp® tube for 25 cycles.

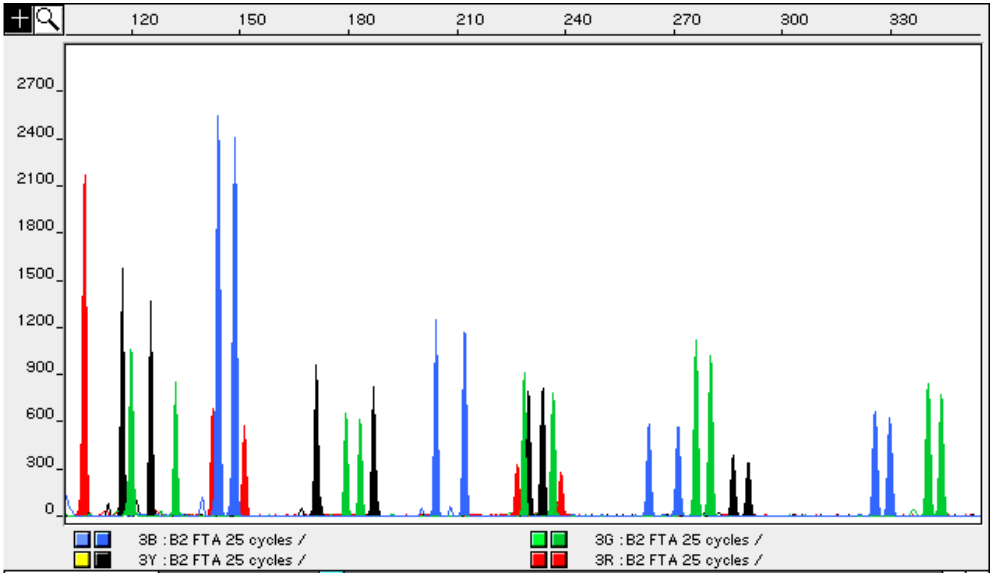


Figure 2-1 AmpFℓSTR Identifiler kit results from a 1.2-mm FTA bloodstain punch (25 cycle amplification), analyzed on the ABI PRISM 310 Genetic Analyzer

Protocol for 310 Genetic Analyzer with Mac OS

3

Overview

About This Chapter

AmpFℓSTR® Identifiler™ PCR Amplification Kit products are electrophoretically separated using a capillary filled with POP-4™ (Performance Optimized Polymer 4) and detected on the ABI PRISM® 310 Genetic Analyzer. Protocols for analyzing samples on the ABI PRISM 310 Genetic analyzer are described in this chapter.

In This Chapter

This chapter contains the following topics:

Topic	See Page
Overview	3-1
Software Requirements	3-2
Preparing the ABI PRISM 310 Genetic Analyzer	3-3
Setting Up the Run	3-11
Filter Set G5 Module Files	3-14
Five-Dye Data Collection	3-15
Making a Matrix File	3-18
Running DNA Samples	3-22
Setting Up Software Parameters	3-26
GeneScan Software Results	3-30
Off-Scale Data	3-33
Shutting Down the Instrument	3-34
Dedicated Equipment and Supplies	3-35

Software Requirements

Collection Software

Data Collection Software v2.1 must be installed before AmpFℓSTR Identifiler PCR Amplification Kit products can be run in order to properly collect five-dye data. Additionally, before running AmpFℓSTR Identifiler PCR products on the instrument, a matrix file must be made using the 6-FAM™, VIC™, NED™, PET™ and LIZ™ matrix standards run using the GS STR POP 4 (1 mL) G5 module.

Analysis Software

This chapter was written for use with GeneScan Analysis 3.1 Software or higher. Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual* (P/N 903565) and *GeneScan Analysis Software Version 3.1 User's Manual* (P/N 403001) for more detailed information on the instrument and software used with these protocols. Genotyper® software v2.5.2 or higher must be used to analyze Identifiler kit data (see Chapter 5).

ABI PRISM 310 Genetic Analyzer

Figure 3-1 shows the ABI PRISM® 310 Genetic Analyzer. The parts mentioned in this section are labeled. Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* (P/N 903565) and *GeneScan® Analysis Software Version 3.1 User's Manual* (P/N 4306157) for more detailed information on the instrument and software used with this protocol.

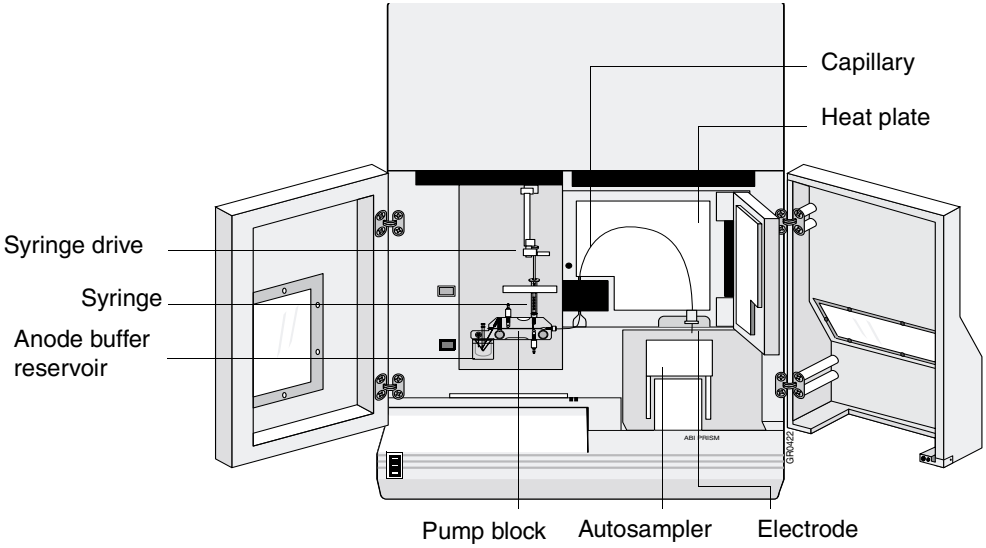


Figure 3-1 ABI PRISM 310 Genetic Analyzer

Installing a New Electrode

Installing and trimming a new electrode is usually necessary only when the instrument is first set up or if the electrode was or has been bent severely.

IMPORTANT A new electrode must be trimmed to the correct length. Refer to Figure 3-2 for trimming the electrode.

Note Not all electrodes need to be trimmed. Trim only as needed.

To install a new electrode:

Step	Action
1	Install the new electrode on the instrument as described in the *ABI PRISM 310 Genetic Analyzer User's Manual*.
2	Under the **Manual Control** menu, select **Home Z-Axis**.
3	a. Use the flush-cutting wire cutter (P/N T-6157) provided in the instrument packing kit. b. Hold the cutters with the flat cutting face toward the top of the instrument.
4	Cut a small amount off the end of the electrode until it is flush with the lower surface of the stripper plate. Be careful not to flex the stripper plate upwards while cutting. Do not cut off more than 1mm beyond the lower surface of the stripper plate (Figure 3-2).

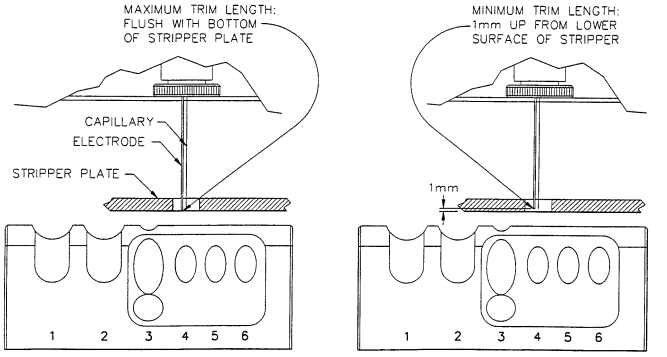


Figure 3-2 Trimming the electrode

Cleaning the Electrode

To clean the electrode:

Step	Action
1	Wipe the electrode with a Kim wipe tissue that has been dampened with distilled, deionized water.
2	Dry the electrode with a fresh Kim wipe tissue. **Note** The autosampler should be recalibrated after cleaning the electrode, as described in "Calibrating the Autosampler" of the *ABI PRISM 310 Genetic Analyzer User's Manual*.

Removing the Syringe

To remove the syringe:

Step	Action
1	Launch the ABI PRISM 310 Data Collection software.
2	a. Under the **Window** menu, select **Manual Control**. b. Select **Syringe Home** from the **Function** pop-up menu. c. Click **Execute**. **Note** For all commands in the **Manual Control** window, the **Execute** button must be selected to complete the task.
3	Open the instrument doors and move the syringe drive toggle to the left.
4	Unscrew the syringe from the pump block.

Checking the Syringe

Verify that the 1.0-mL glass syringe (P/N 4304471) has a small O-ring (P/N 221102) inside the syringe, and that another O-ring is placed around the ferrule-shaped seal. The ferrule should be firmly seated in the end of the 1.0-mL syringe. If the syringe is dirty, it must be cleaned before use.

Cleaning the Syringe

To clean the syringe:

Step	Action
1	Remove the plunger by slowly drawing it from the glass barrel (count to 5, this should take approximately 5 sec.) while keeping the entire syringe submerged in water. **IMPORTANT** Moving the dry plunger quickly can damage it, resulting in premature failure or leakage around the plunger.

To clean the syringe:

Step	Action
2	Remove the ferrule from the syringe. a. Soak the ferrule in warm (not boiling) water for as long as it takes to remove crystals (if any) in the ferrule. b. Rinse the ferrule with deionized water.
3	Clean the glass barrel with warm water. Dissolve any crystals.
4	Rinse the glass barrel with distilled, deionized water. **IMPORTANT** Remove all residual water from the syringe by blowing compressed air through it.
5	Inspect the O-ring in the stainless steel hub of the syringe for damage, and replace it if necessary. **IMPORTANT** Make sure the O-ring does not block the hole in the stainless steel hub.
6	Inspect the O-ring on the ferrule and replace it if necessary.
7	Place the ferrule back onto the syringe. **IMPORTANT** The Teflon™ tip of the plunger must be damp when inserting it into the barrel (place a drop of distilled deionized water on the Teflon), or the Teflon™ tip could be damaged. **Note** For syringe storage, the plunger should remain in the syringe barrel.

Loading the Syringe

To load the syringe:

Step	Action
1	Prime the syringe with approximately 0.1 mL of POP-4 polymer.
2	Fill the 1.0-mL syringe manually with a maximum of 0.8 mL of POP-4 polymer. **Note** The polymer should not stay in the syringe longer than 3 days. Do not return unused polymer to the bottle. **Note** Before use, the POP-4 polymer should be allowed to equilibrate to room temperature. If precipitate is present in the bottle when removed from cold storage, it should go back into solution at room temperature. Gently mix the polymer thoroughly by inversion before using.
3	Wipe the outside of syringe with a Kim wipe tissue to dry.
4	Remove any air bubbles by inverting the syringe and pushing a small amount of polymer out of the tip.

Removing and Cleaning the Pump Block

Before setting up the instrument for a run, make sure that the pump block is clean of all polymer, especially if the polymer in the syringe has been sitting at room temperature for more than three days. Urea decomposition during this interval causes transient current increases (spikes) during electrophoresis.

To remove and clean the pump block, see “Cleaning and Maintaining the Instrument” in the *ABI PRISM 310 Genetic Analyzer User’s Manual*. Follow the instructions in the sections titled “Removing the Pump Block,” “Rinsing the Pump Block,” and “Replacing the Pump Block.” We do not recommend following the section titled “Rinsing the Pump Block on the Instrument” for this application.

IMPORTANT Remove all residual water from the pump block and fittings by blowing canned compressed air through the channels. Make sure the can is held upright or the propellant in the can may be shot into the gel block. This can result in poor resolution or high baseline.

Reinstall the pump block on the instrument after cleaning.

Installing the Syringe on the Pump Block

To install the syringe on the pump block:

Step	Action
1	Move the syringe drive toggle on the instrument to the left in order to be able to attach the syringe to the pump block.

To install the syringe on the pump block: *(continued)*

Step	Action
2	Place the 1.0-mL syringe through the right-hand port of the plastic syringe guide plate and screw the syringe into the pump block. The syringe should be finger-tight in the block.
3	Hand-tighten the valves on the pump block to the left of and below the syringe. **Note** Overtightening can cause microscopic fractures in the pump block. Undertightening may result in "syringe leak detected" message.

Installing the Capillary

To install the capillary :

Step	Action
1	a. Clean capillary window with 95% ethanol on a lint-free tissue. b. Do not touch capillary window after cleaning.
2	a. Install the 47-cm, 50-µm i.d. capillary (P/N 402839, green mark) as described in the *ABI PRISM 310 Genetic Analyzer User's Manual*. b. Follow the instructions in the section titled "Installing the Capillary." c. If a new capillary has been installed, select **Change Capillary** under the Instrument menu. d. Select **OK** in the Reset window to set the injection counter to zero.
3	After installing the capillary, secure it into place by pressing a piece of thermal tape over it onto the heat plate just above the electrode. **Note** The capillary should be approximately flush with, or less than 1 mm below, the end of the electrode.
4	♦ Calibrate the autosampler. ♦ Make sure that it is calibrated in the X, Y, and Z directions. The capillary should almost touch the metal calibration points. Refer to "Calibrating the Autosampler" in the *ABI PRISM 310 Genetic Analyzer User's Manual*. **IMPORTANT** The sample tray must be removed before calibrating the autosampler. If the sample tray is not removed, the electrode may bend.

Filling the Buffer Reservoirs

To fIll the buffer reservoirs:

Step	Action
1	Dilute 5 mL of 10X Genetic Analyzer Buffer with EDTA (P/N 402824) to 1X concentration (50 mL) with distilled, deionized water. Change to fresh buffer every 48 hours or 96 injections, whichever comes first.
2	a. Fill the anode buffer reservoir to the red line with 1X Genetic Analyzer Buffer. b. Secure the reservoir on the pump block. **⚠ CAUTION CHEMICAL HAZARD. 10X Genetic Analyzer Buffer with EDTA.** May cause eye, skin and respiratory tract irritation. Please read the MSDS, and follow handling instructions. Wear appropriate protective eye wear, clothing, and gloves.
3	a. Fill a 4-mL glass buffer vial (P/N 401955) to the fill line with 1X Genetic Analyzer Buffer. b. Insert the plastic vial lid with attached septum (P/N 402059) into the glass vial. c. Place the buffer vial into position 1 on the autosampler. This will serve as the cathode buffer. **Note** Overfilling and underfilling one or both buffer reservoir and vial can cause siphoning. Pay close attention to the red fill line.
4	a. Fill a second 4-mL glass buffer vial to the fill line with distilled water. b. Insert the plastic vial lid with attached septum into the glass vial. c. Place the vial into position 2 on the autosampler.
5	a. Fill a 1.5-mL Eppendorf tube full with distilled water. b. Place it into position 3 on the autosampler. **Note** Do not use a screw-cap tube. The lids on screw-cap tubes are too high to clear the electrode and capillary. Use a 1.5-mL Eppendorf tube with the lid clipped off.

Priming the Pump Block

To prime the pump block:

Step	Action
1	a. From the **Window** menu, select **Manual Control**. b. Select **Buffer Valve Close** from the pop-up menu. c. Click **Execute**.
2	Partly unscrew the capillary filling ferrule.

To prime the pump block: *(continued)*

Step	Action
3	Manually press down on the 1.0-mL syringe plunger until the ferrule space is filled with polymer. **Note** This will remove the air bubbles at the ferrule site.
4	Tighten the ferrule to close.
5	Partly unscrew the waste valve on the pump block (below the syringe).
6	Manually press down on the 1.0-mL syringe plunger until the valve space is filled with polymer. **Note** This will remove the air bubbles at this valve site, and should use about 0.1 mL of polymer.
7	Tighten the waste valve to close.
8	To open the pin valve at the anode buffer reservoir on the pump block, a. From the **Manual Control** window, select **Buffer Valve Open.** b. Click **Execute**.
9	Manually press down on the 1.0-mL syringe plunger to push enough gel through the block so that all of the air bubbles are removed from the polymer channel in the block. (This process should use about 0.2 mL of polymer). **IMPORTANT** There should be no air bubbles in the pump block channels.
10	a. Close the pin valve by selecting **Buffer Valve Close** from the pop-up menu in the **Manual Control** window. b. Click **Execute**.
11	Move the syringe drive toggle to the right so that it is positioned over the syringe plunger.
12	a. From the **Manual Control** window select **Syringe Down**. b. Select 50-step intervals. Execute until the toggle almost makes contact with the syringe plunger. c. Click **Execute**. d. Select smaller step intervals until the toggle makes contact with the syringe plunger.

Setting Up the Run

Setting the Run Temperature

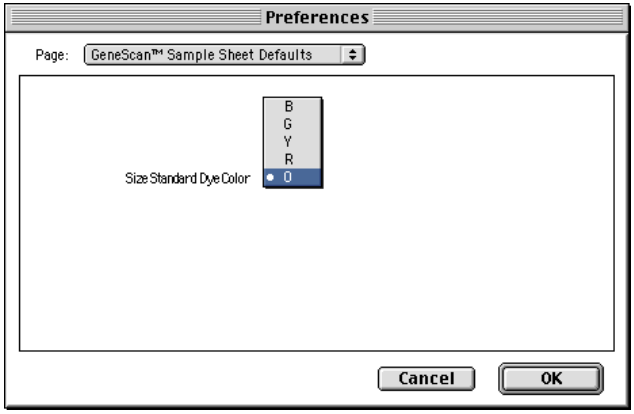
Setting the run temperature prior to starting a run is optional; however, this step saves time. This heating step occurs automatically at the beginning of the GS STR POP4 (1 mL) G5 run module.

To set the run temperature:

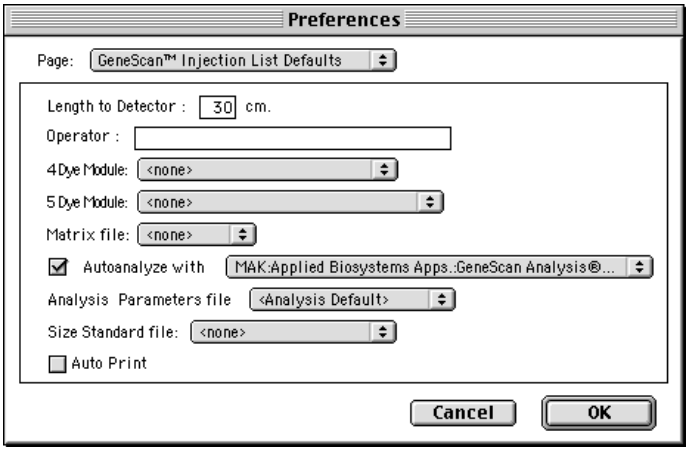
Step	Action
1	Close the instrument doors.
2	Return to the ABI PRISM 310 Collection Software.
3	a. From the Window menu, select **Manual Control**. b. Select **Temperature Set** from the pop-up menu.
4	a. Set the temperature to 60 °C. b. Click **Execute**. **Note** It takes up to 30 min for the instrument to reach the 60 °C run temperature. Samples can be prepared while the instrument is heating.

Setting the Parameters

To choose a five-dye sample sheet:

Step	Action
1	This is an optional step: Launch the 310 Genetic Analyzer Data Collection software v2.1. From the **Window** menu, under **Preferences,** choose the **GeneScan™ Sample Sheet Defaults.** Set the size standard color to orange (O) as shown in the figure. 

To choose a five-dye sample sheet: *(continued)*

2	Select **GeneScan Injection List Defaults** from the **Page** drop-down menu. The following window appears. 
3	Make the following selections in the above window: a. Select GS STR POP4 (1 mL) G5 for the five-dye module. b. Choose a default matrix file. c. Make sure the Genescan Analysis application is selected if you wish to autoanalyze. If you do not wish to autoanalyze your data, deselect the box next to the **Autoanalyze with** option. **Note** When you create a new sample sheet, a portion of the form is automatically filled in for you. You can modify the automatic defaults in the Preferences file.
4	Once you have finished making changes to the **Preferences** pages, click **OK** to save your changes.

Running Matrix Samples

The precise spectral overlap between the five dyes is measured by analyzing DNA fragments labeled with each of the dyes (6-FAM, VIC, NED, PET or LIZ dye) in separate injections on a capillary. These dye-labeled DNA fragments are called matrix standard samples. See Chapter 1 for a general description of multicomponent analysis.

The ABI PRISM® GeneScan® Analysis Software v3.1 or higher analyzes the data from each of these five samples and creates a matrix file. The matrix file contains a table of numbers with five columns and five rows. These numbers are normalized fluorescence intensities and represent a mathematical description of the spectral overlap that is observed between the five dyes (Figure 3-3 on page 3-13).

The rows in the matrix file table represent the virtual filters and the columns represent the dye-labeled DNA fragments, indicated as

"Reactions" in Figure 3-4 on page 3-19. The top left-hand value, 1.0000, represents the normalized fluorescence of blue (6-FAM-labeled) DNA fragments in the blue filter. It follows that all matrix tables should have values of 1.0000 on the diagonal from top left to bottom right, as shown in Figure 3-3.

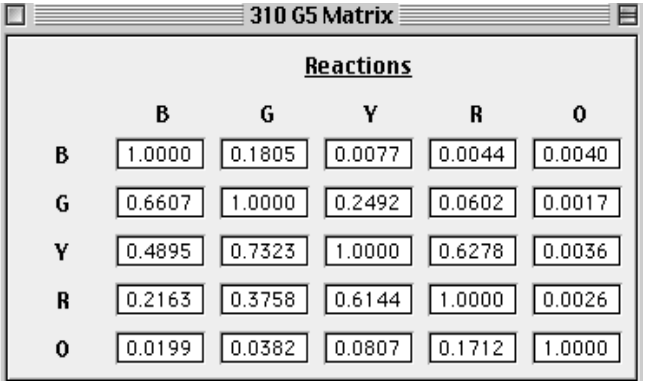


Figure 3-3 Matrix file table, indicating the values obtained on a particular ABI PRISM 310 System. The values obtained will be unique for each instrument.

The other values in Figure 3-3 should all be less than 1.0000. These values represent the amount of spectral overlap observed for each dye in each virtual filter. For example, the values in the first column reflect quantitatively the amount of blue dye detected in each virtual filter. These matrix file values will vary between different instruments, virtual filter sets, and run conditions on a single instrument. A matrix file must be made for each instrument and for a particular set of run conditions.

The appropriate matrix file can be applied to data on subsequent runs on the same instrument, as long as the running conditions are constant from run to run. This is because the spectral overlap between the five dyes is reproducible under constant run conditions. However, it is recommended that a new matrix be made once a month for use with the AmpFℓSTR products or when changing lots of polymer, capillaries, and buffer.

Multicomponent analysis is accomplished automatically by the GeneScan Analysis software, which applies a mathematical matrix calculation (using the values in the matrix file) to all sample data.

Filter Set G5 Module Files

Overview

The ABI PRISM® 310 Data Collection Software v2.1 collects light intensities from five specific areas on the CCD camera, each area corresponding to the emission wavelength of a particular fluorescent dye. Each of these areas on the CCD camera is referred to as a "virtual" filter since no physical filtering hardware (*e.g.*, band pass glass filter) is used.

The information that specifies the appropriate virtual filter settings for a particular set of fluorescent dyes is contained in each approprate ABI PRISM Data Collection Software module file.

The module file that must be installed and used for dye set DS-33 (6-FAM™, VIC™, NED™, PET™, LIZ™ dyes) on the ABI PRISM 310 Genetic Analyzer is the following:

Instrument	Configuration	Module File
ABI PRISM 310	POP-4™ polymer with 1-mL syringe	GS STR POP4 (1 mL) G5

IMPORTANT Filter Set G5 module files must be installed on the instrument's computer before making a matrix file using the 6-FAM, VIC, NED, PET, and LIZ matrix standards. Filter Set G5 module files must also be used on all subsequent runs. Samples that are run on a capillary using Filter Set G5 must be analyzed using a matrix file that was created using Filter Set G5.

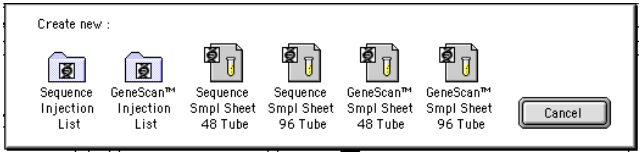
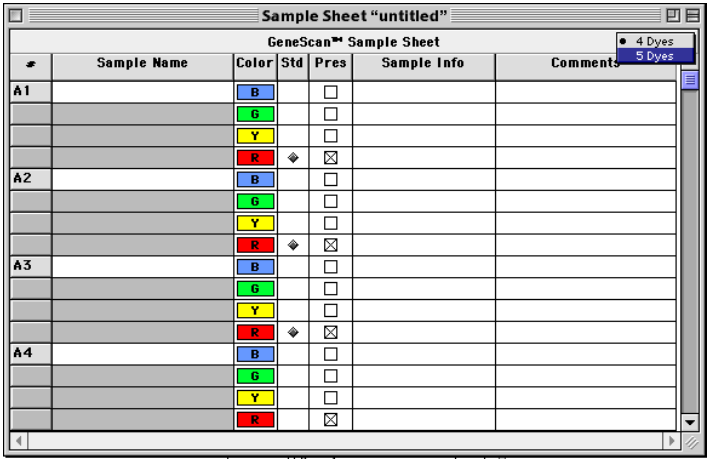
Five-Dye Data Collection

Overview

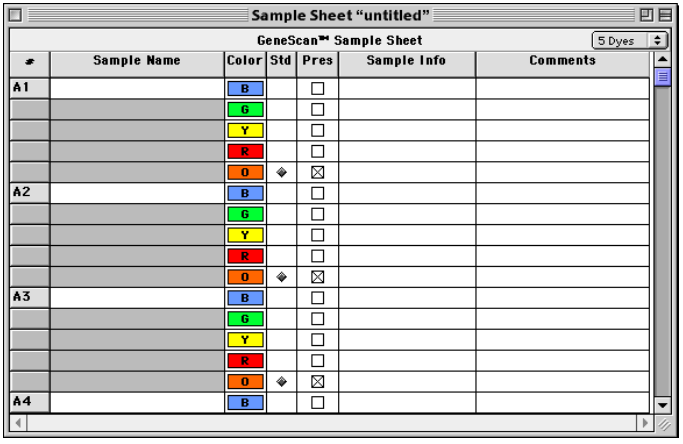
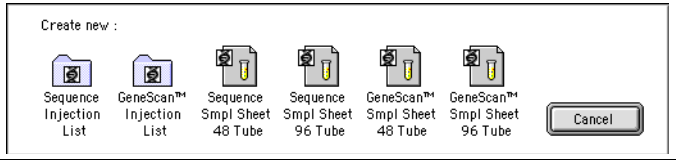
The ABI PRISM® 310 Data Collection Software v2.1 enables collection of five-dye data for DNA fragment analysis applications. This section provides detailed information on sample sheet and injection lists.

Creating a Five-Dye Sample Sheet and Injection List

To create a five-dye sample sheet:

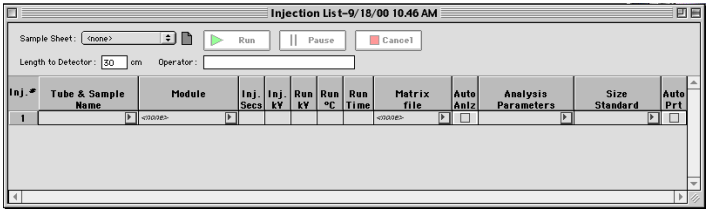
Step	Action
1	From the **File** menu, choose **New**. The **Create new** window appears. 
2	Choose the icon corresponding to an appropriate GeneScan Sample Sheet configuration. A Sample Sheet window appears. 
3	Choose the **5 Dyes** option from the drop-down menu in the upper-right corner of the window.

To create a five-dye sample sheet: *(continued)*

Step	Action
4	In the five-dye Sample Sheet: Enter sample name, sample information and comments. Designate color for appropriate size standard. Save. Be sure to select the orange dye as the designated size standard for all five-dye samples. Under Preferences, this feature can be preset. See page 3-11.  Setting up five-dye samples requires the use of a five-dye sample sheet. You may not set up both four-dye and five-dye samples in a five-dye sample sheet. All four-dye samples must be set up separately in a four-dye specific sample sheet.
5	To create a new injection list, choose **New** from the **File** menu. The **Create new** window appears. 
6	Choose the **GeneScan™ Injection List** icon.

Sample Sheet "untitled"
GeneScan™ Sample Sheet
5 Dyes
Sample Name Color Std Pres Sample Info Comments
A1 B G Y R O
A2 B G Y R O
A3 B G Y R O
A4 B

To create a five-dye sample sheet: *(continued)*

Step	Action
7	From the Sample Sheet drop-down menu (in the GeneScan Injection List), import the appropriate sample sheet. **Note** To access five-dye modules, you must first import a five-dye sample sheet into the injection list. 
8	After setting the appropriate injection parameters, save the injection list.
9	To start the sequence of injections, click the **Run** option in the **Injection List** window.

Making a Matrix File

Matrix Standards

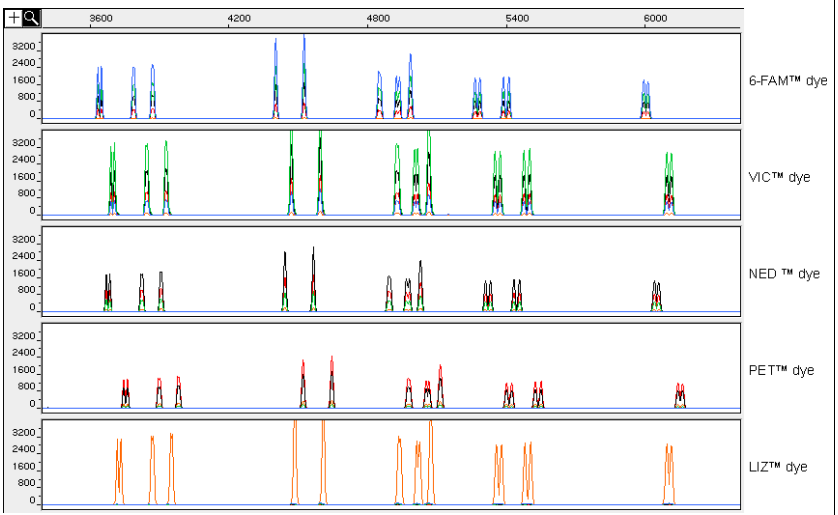
The matrix standards are supplied in the Matrix Standard Set DS-33 (6-FAM™, VIC™, NED™, PET™ and LIZ™) for use with the 310/377 system (P/N 4318159).

Making a Matrix File on the ABI PRISM 310

To make the matrix file:

Step	Action
1	♦ Combine 1 µL of each matrix standard with 25 µL of Hi-Di™ Formamide (P/N 4311320). ♦ Prepare one tube for each matrix standard sample. **⚠ WARNING CHEMICAL HAZARD. Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. **IMPORTANT** Do not include the GeneScan-500 LIZ Size Standard in the preparation of the matrix standards.
2	a. Denature the samples at 95 °C for 3 min. b. Quick chill on ice for 3 min. c. Place tubes in the appropriate sample tray. **Note** Be careful not to carry-over any water on the outside of the tubes. Water on the autosampler tray may promote arcing.
3	Launch the ABI PRISM 310 Collection application.
4	Under the **File** menu, select **New** and click the **GeneScan Smpl Sheet 48 Tube** or **GeneScan Smpl Sheet 96 Tube** icon, as appropriate.
5	a. Complete the sample sheet as described in the *ABI PRISM 310 Genetic Analyzer User's Manual*. b. Enter the sample names/numbers for each row in the **Sample Name** column. This will identify which sample is in which tube of the sample tray. c. **Close** and **Save** the sample sheet.
6	Under the **File** menu, select **New** and click the **GeneScan Injection List** icon.

To make the matrix file: *(continued)*

Step	Action
7	a. In the **Injection List**, select the appropriate sample sheet from the **Sample Sheet** pop-up menu. b. From the **Module** pop-up menu, choose GS STR POP4 (1 mL) G5 for every injection. c. Choose **None** in the **Matrix File** column for each matrix standard sample. **Note** Review data of each matrix standard. Re-inject if necessary.
8	Click **Run**.
	 **Figure 3-4** This figure exhibits the raw data of each matrix standard, analyzed on the ABI PRISM 310 Genetic Analyzer.
9	When the injections are done, follow these steps in the GeneScan Analysis Software: a. Under the **File** menu, select **New**. b. Click the **Matrix** icon. Select five dyes from the number of dyes pop-up window. In the window that appears, indicate the sample files that correspond to each matrix standard dye color. c. Select starting scan numbers for each sample to exclude the primer peak, as represented in Figure 3-4. d. Select the number of points such that at least these five peaks are contained in the scanned region (this is approximately 2500 scan data points). Avoid spikes or artifacts if possible when selecting the range. e. Click **OK**. The computer makes the matrix and the matrix file table appears.

To make the matrix file: *(continued)*

Step	Action
10	Save the matrix file in the ABI folder within the System folder.

To verify the accuracy of the matrix file:

Step	Action
1	Apply the new matrix file to the Matrix Standard Sample Files as follows: a. In the **Analysis Control** window, highlight the **Sample File** column by clicking in the **Sample File** title row. b. Under the **Sample** menu, select **Install New Matrix**. c. Choose the new matrix file (located in the ABI folder within the System folder) and click **Open**.
2	Analyze the matrix standard samples as follows: a. Under the **Settings** menu, select **Analysis Parameters**, and verify that the settings are correct. b. In the **Analysis Control** window, select all five colors in each sample row for all of the matrix standard samples. c. Click the **Analyze** button.

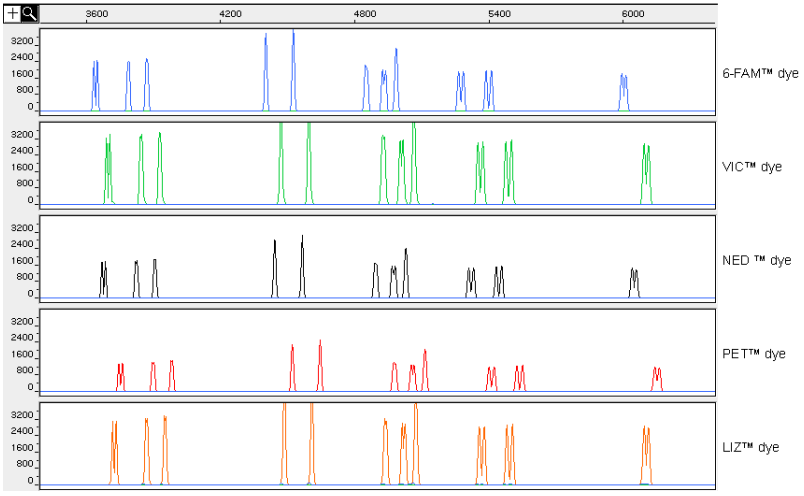


Figure 3-5 This figure exhibits the analyzed data of each matrix standard, analyzed on the ABI PRISM 310 Genetic Analyzer.

To verify the accuracy of the matrix file: *(continued)*

Step	Action
3	a. In the **Results Control** window, examine the results for all five colors for each of the matrix standard samples. For example, the 6-FAM matrix standard results should have peaks for Blue. Evaluate the baseline. A pattern of pronounced peaks or dips in any of the other four colors indicates that the color separation may not be optimal. Examine the results for each matrix standard sample in this way (see Figure 3-5).
4	If this verification test fails, then the capillary may not have been aligned properly in the instrument during the run. Do the following: a. Repeat the experiment, making sure that the capillary is placed carefully in the laser detection window. b. Tape the capillary to the heat plate so that the capillary is immobilized during the run.

Once a satisfactory matrix file has been made, this matrix file can be applied to subsequent runs. It is not necessary to run matrix standard samples for each new capillary.

Preparing Samples and AmpFℓSTR Identifiler Allelic Ladder

To prepare the samples:

Step	Action
1	Combine the necessary amount of Hi-Di™ Formamide and GeneScan™-500 LIZ™ Size Standard (P/N 4322682) in a single microcentrifuge tube as shown: ♦ (Number of samples + 2) × 24.5 µL Hi-Di Formamide ♦ (Number of samples + 2) × 0.5 µL GeneScan-500 LIZ Size Standard If you are using a multi-channel pipettor or processing many samples, you may want to prepare additional master mix. **⚠ WARNING CHEMICAL HAZARD**. **Formamide** is harmful if absorbed through the skin and may cause irritation to the eyes, skin, and respiratory tract. It may cause damage to the central nervous system and the male and female reproductive systems, and is a possible birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eye wear, clothing, and gloves. Be sure to include at least one injection of AmpFℓSTR® Identifiler™ Allelic Ladder per run in the calculations.
2	a. Vortex the tube to mix. b. Spin the tube briefly in a microcentrifuge.
3	a. Label tubes as appropriate. b. Aliquot 25 µL of Hi-Di Formamide/GeneScan-500 LIZ solution into 0.2-mL or 0.5-mL Genetic Analyzer sample tubes. **Note** To pipet the Hi-Di Formamide/size standard solution, we recommend using a repeating pipettor.
4	Add 1.5 µL of PCR product or AmpFℓSTR Identifiler Allelic Ladder per tube. Mix by pipetting up and down.
5	Seal each tube with a septum.
6	Vortex the sample tray and spin briefly in a microcentrifuge. **Note** Ensure that there are no bubbles.
7	Denature each sample for 3 min at 95 °C.
8	Chill tubes for at least 3 min on ice. **Note** Be careful not to carry-over any water on the outside of the tubes. Water on the autosampler tray may promote arcing.

Loading Samples

To load samples:

Step	Action
1	Open the instrument door and press the **Tray** button to present the autosampler.
2	Place a 48-well or 96-well sample tray on the autosampler. For a 48-well autosampler tray, tube #1 will go into sample tray position A1, tube #2 into sample tray position A3, etc. For a 96-well autosampler tray, tube #1 will go into sample tray position A1, tube #2 into sample tray position A2, etc.
3	Press the **Tray** button on the instrument to retract the autosampler.
4	Close the instrument door.

Sample Electrophoresis

To run the samples:

Step	Action
1	If not already open, launch the ABI PRISM 310 Data Collection Software v2.1.
2	From the **File** menu, select **New** and click the appropriate **GeneScan Smpl Sheet** icon. **Note** The 310 Genetic Analyzer Data Collection Software v 2.1 must be installed for use with the AmpFℓSTR Identifiler PCR Amplification Kit.
3	Complete the sample sheet. The sample sheet can be prepared at any time before the preparation of samples and saved in the Sample Sheet folder. a. Select 5-dyes from the drop-down menu. b. Enter sample names/numbers for each injection in the **Sample Name** column. This will later indicate which sample is in which tube of the sample tray. c. Enter the sample description for each row in the **Sample Info** column (for Blue, Green, Yellow and Red for each sample). This is necessary for the AmpFℓSTR® Identifiler™ Template File to build tables containing the genotypes for each sample. Type the word **Ladder** for the Blue, Green, Yellow and Red rows for the AmpFℓSTR® Identifiler™ Allelic Ladder injection. **Note** Software requires the word “Ladder.” See page 5-10. **Note** Alternatively, a. Select 5-dyes from the drop-down menu. b. Enter the sample names and numbers for each injection in the **Sample Name** column. c. Using copy feature under **Edit** menu, copy all sample names at one time by highlighting the “Sample Name” header and paste by highlighting the “Sample Info” header. The sample name will appear in the blue, green, yellow, red and orange Sample Info column for each sample.
4	From the **File** menu, select **New** and click on the **GeneScan Injection List** icon.
5	Choose the appropriate sample sheet from the **Sample Sheet** pop-up menu (at the top left of the **Injection List** window).

To run the samples: *(continued)*

Step	Action
6	From the **Module** pop-up menu, choose **Module GS STR POP4 (1 mL) G5** for every injection. a. Click the arrow in the Module column for the first sample/injection to view the pop-up menu and choose the GS STR POP4 (1 mL) G5 module file. b. Select the entire Module column by clicking the **Module** column heading and choosing Fill Down (from the **Edit** menu). **Note** This function will not need to be performed if the preferences were set as described on page 4-12.
7	From the **Matrix** file pop-up menu, choose the appropriate matrix file for every injection. a. Click the arrow in the **Matrix** column for the first sample/injection to view the pop-up menu and choose the appropriate matrix file. Select the entire Matrix column by clicking the Matrix column heading and choosing **Fill Down** (from the **Edit** menu). **IMPORTANT** The matrix file must be one that was made using the 6-FAM, VIC, NED, PET and LIZ matrix standards and Filter Set G5 module. Furthermore, if you wish to autoanalyze, a copy of the matrix file must be placed in the ABI folder located in the System Folder.
8	Click the **Run** button. **Note** If you have not preheated the heat plate, the module has an initial step in which the plate is heated to 60 °C before running the first sample. This step takes up to 30 min. Once the plate reaches 60 °C, the run will begin.

Setting Up Software Parameters

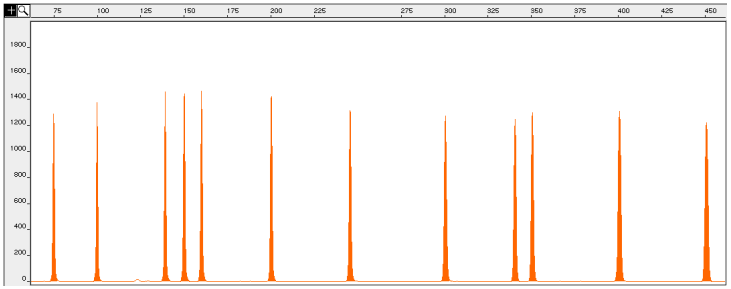
Setting the Analysis Parameters

Perform the following steps in GeneScan Software v3.1 or higher.

To set the analysis parameters:

Step	Action
1	Launch the GeneScan Software v 3.1.
2	From the **Settings** menu, select **Analysis Parameters**. **Note** A more detailed discussion can be found for each of the six "Analysis Parameters" in the *GeneScan Analysis 3.1 User's Manual*.
3	Fill in the dialog box. a. **Analysis Range:** – Choose **This Range (Data Points)** radio button. – Enter Start and Stop data point numbers in the entry fields. The **Start** data point should be a selected data point just before the first peak of interest, the 75 bp size standard peak. At a minimum, the **Stop** data point should be a selected data point just after the last peak of interest, the 450 bp size standard peak. – Look at the raw data and enter the values that will be appropriate for all sample files in the project. These data points will affect what data is displayed in the results display. b. **Data Processing:** – The **Baseline** and the **MultiComponent** check boxes should be selected. – Choose a **Smooth Option.** Smooth Options can affect peak height and peak definition. – The "Light smoothing option" is recommended for use with the AmpFℓSTR products on the Macintosh® computer. c. **Peak Detection:** – Choose a Peak Amplitude Threshold (PAT) for each dye color. – Use the active scroll bar to input the PATs for each of the five colors. – After analysis, the GeneScan table will contain data for all peaks with a height above that specified by the PAT.

To set the analysis parameters: *(continued)*

Step	Action
	Note We suggest that you determine the PATs appropriate for your analysis. Sensitivity experiments should be conducted in your laboratory with each instrument to evaluate the PATs used for analysis. – The Min Peak Half Width for use with the AmpFℓSTR products is 3 Pts. d. **Size Call Range:** Choose the **This Range (Base Pairs)** radio button and enter the value of **75** for Min and **450** for Max. e. **Size Calling Method:** Choose the **Local Southern Method** radio button for sizing of the AmpFℓSTR products. This method determines the sizes of fragments by using the reciprocal relationship between fragment length and mobility. f. **Split Peak Correction:** Choose the **None** radio button; no correction is needed for use with the AmpFℓSTR products.
4	Click **OK** when done.
5	Assign a size standard: a. Click the arrow in the Size Standard column for a sample file to view the pop-up menu and select **Define New**. – For more information on defining a size standard, refer to the *GeneScan Analysis 3.1 User's Manual*. – Do not assign a size for the 250-bp peak for data generated on the ABI PRISM® 310 Genetic Analyzer (*i.e.*, assign a size of zero). This peak can be used as an indicator of precision within a run. Twelve size standard peaks should be viewed at this step, as shown below.  – Save the size standard for this sample in the GS Standards Folder within the ABI PRISM GeneScan Version 3.1 Software folder. b. To apply this standard to all injections, choose the appropriate standard in the Size Standard column header (above sample 1) in the **Analysis Control** window.

To set the analysis parameters: *(continued)*

Step	Action
6	Analyze sample files: a. Highlight the blue, green, yellow, red and orange columns. **Note** Confirm that the orange box has been indicated as the standard (a diamond symbol should appear in all orange boxes where a size standard is included with the sample files). If the diamond symbol is not in the orange boxes, ⌘ -mouse click will place a diamond in the box. b. Click the **Analyze** button.
7	After the analysis is complete, confirm that the sizes for the peaks in the GeneScan™-500 LIZ™ Size Standard have been correctly assigned. a. Open **Results Control** (from the **Window** menu) and examine the orange GeneScan-500 LIZ Size Standard peaks in overlapping groups of 16 samples (Quick Tile Off). Be sure to use the **Align By Size** option under the **View** menu. b. While the samples are tiled, check the 250-bp peaks (sized as approximately 246 bp) in the enlarged view window. Remember that this peak was not defined in the size standard. The tiled 250-bp peaks should size consistently, *i.e.*, should all overlap. In a typical run, the 250-bp peaks all fall within a size window of approximately 1 bp. Temperature fluctuations in the laboratory may cause variations > 1 bp. **Note** Laboratory temperature variations can cause size shifts. If the temperature of the laboratory varies, try injecting the AmpFℓSTR Identifiler Allelic Ladder approximately every 10 injections, or 5 hours. c. Scroll through the tables to verify correct GeneScan-500 LIZ peak assignments. d. Check the GeneScan-500 LIZ Size Standard peaks in the remaining samples, taking note of which samples (if any) have incorrect peak assignments.
8	If the size standard peak assignments are incorrect for one injection, define a new size standard for that sample using the peaks in that injection. To do so, select the **Define New** option in the **Size Standard** pop-up menu for that sample.
9	Re-analyze any incorrectly sized samples (select the blue, green, yellow, red and orange boxes) using the newly defined GeneScan-500 LIZ Size Standard file. This creates a new standard file for each of these samples, replacing the previous analysis results for those samples only.

To set the analysis parameters: *(continued)*

Step	Action
10	Confirm that the GeneScan-500 LIZ Size Standard peaks are now correctly assigned in the re-analyzed samples.
11	View AmpFℓSTR Identifiler kit results (using the Results Control window). Refer to the *GeneScan Analysis 3.1 User's Manual* for printing options.

GeneScan Software Results

GeneScan Analysis Software

After the sample files have been analyzed, the Results Control window is used to display the results from each lane of a gel or each injection into a capillary. The Results Control window displays the newly analyzed sample files and allows the user to specify the format of the results. Selecting both the Electropherogram and Tabular Data icons is recommended for reviewing the results. For more information on displaying the results, refer to the *GeneScan Analysis Software v3.1 User's Manual* (P/N 903565).

Information Provided in the Electropherogram and Table

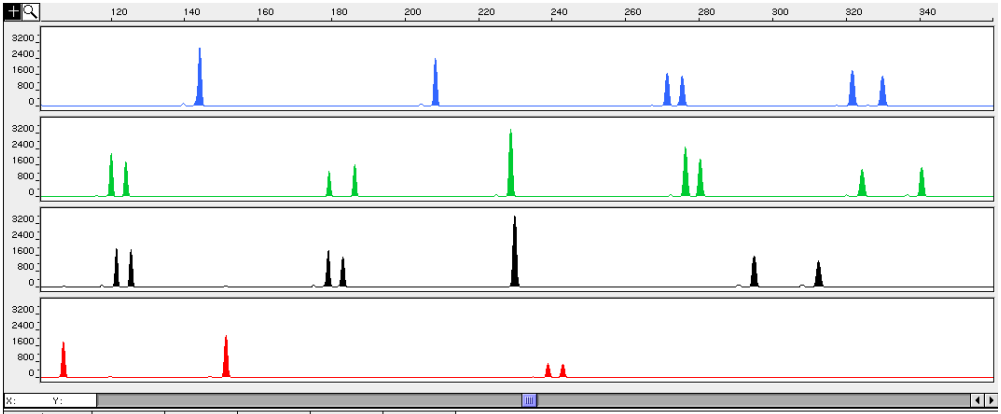
Both the electropherogram and the tabular data can be displayed, see Figure 3-6 on page 3-31.

The electropherogram is a chromatographic display with fluorescence intensity indicated as relative fluorescence units (RFU) on the y-axis. After the internal lane size standard has been defined and applied, the electropherogram can be displayed with the base pair size on the x-axis.

Peaks of all heights within the Analysis Range specified in the Analysis Parameters are displayed on the electropherogram, but those peaks below the Peak Amplitude Threshold (minimum peak height) that are defined in the Analysis Parameters will not be listed in the tabular data.

The columns in the table list the following:

- Column 1 lists the Dye/Sample and Peak (*e.g.*, "4B, 1" indicates the first blue peak in project sample 4).
- Column 2 lists the time it took the dye-labeled fragment to reach the detector
- Column 3 lists the base pair size of the peak, as calculated using the GeneScan™-500 LIZ™ Size Standard curve.
- Column 4 lists the height in RFU of the peak.
- Column 5 lists the relative peak area, which is the integral of the RFU times the data point (scan number). This value depends on the velocity of the dye labeled fragment as it passes the detector.
- Column 6 lists the data point (scan number) of the dye labeled fragment at its maximum peak height; the data point correlates with the number of laser scans (or data points collected) from the beginning of the run until the time that the peak maximum is detected.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
9B, 13	15.40	144.24	3023	22237	4200
9B, 15	17.52	208.34	2433	17040	4778
9B, 17	19.55	271.38	1671	13624	5331
9B, 18	19.68	275.52	1527	12961	5366
9B, 21	21.05	321.86	1799	15800	5739
9B, 23	21.29	329.97	1533	13525	5804
9G, 15	14.59	120.21	2181	15275	3978
9G, 16	14.73	124.17	1802	11915	4016
9G, 18	16.55	179.47	1303	8697	4512
9G, 20	16.78	186.43	1648	10929	4576
9G, 22	18.20	228.84	3523	25880	4962
9G, 25	19.70	276.35	2553	20711	5373
9G, 26	19.83	280.39	1921	15366	5407
9G, 28	21.12	324.49	1379	12208	5760
9G, 31	21.61	340.66	1492	13626	5892
9Y, 6	14.64	121.56	1996	11921	3991
9Y, 7	14.78	125.53	1957	11818	4029
9Y, 10	16.54	179.25	1857	13356	4510
9Y, 12	16.67	183.17	1581	10615	4546
9Y, 14	18.23	229.97	3673	29655	4972
9Y, 16	20.28	295.03	1603	13587	5529
9Y, 18	20.78	312.56	1364	11795	5666
9R, 1	14.12	107.16	1838	13696	3850
9R, 7	15.63	151.40	2153	15070	4261
9R, 9	18.53	239.04	750	5650	5052
9R, 10	18.66	243.03	687	5429	5087

Figure 3-6 GeneScan electropherogram of AmpFℓSTR Identifiler alleles in AmpFℓSTR Control DNA 9947A analyzed on the ABI PRISM 310 Genetic Analyzer

Results Display Options

The GeneScan Software v3.1 or higher offers two main options in the Results Control window for electropherogram viewing formats: Quick Tile Off and Quick Tile On.

- The “Quick Tile Off” format provides the option of displaying results either for multiple colors within a single lane or injection or from multiple lanes or injections in the same panel, (*i.e.*, the results are overlaid). This is demonstrated in panel 1 of Figure 3-7.
- The “Quick Tile On” format displays each color of each lane or injection separately, as shown in panels 2–5 of Figure 3-7.

The Quick Tile Off and On feature offers the user versatility in customizing the display of results. Up to eight panels can be tiled at a single time and up to 16 electropherogram may be overlaid in one panel at the same time.

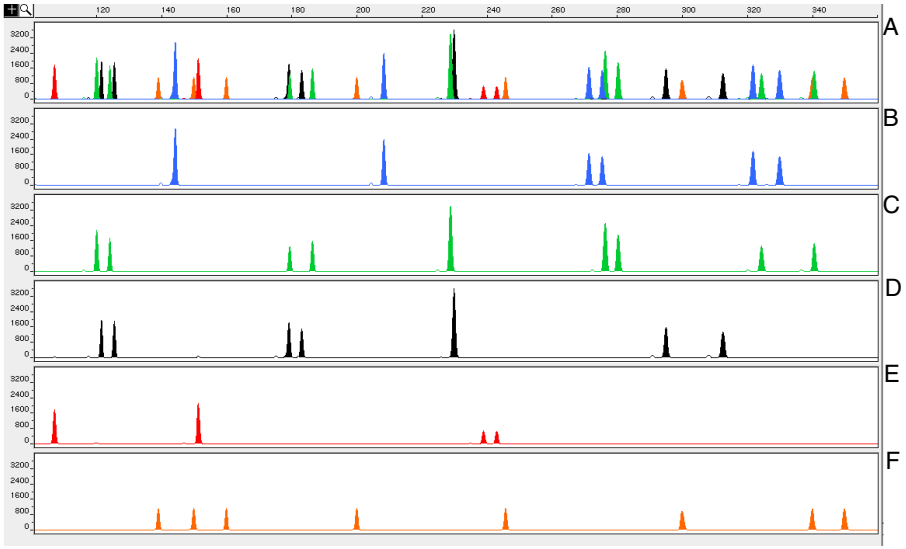


Figure 3-7 Quick Tile Off and Quick Tile On options. Panel A is an example of one sample displayed with Quick Tile Off. Panels B–F are examples of the same sample file with Quick Tile On using the AmpFℓSTR Control DNA 9947 analyzed on the ABI PRISM 310 Genetic Analyzer

Note For a more detailed description see the *GeneScan Software v3.1 User's Manual*.

Standards for Samples

For the Identifiler kit, the panel of standards needed for PCR amplification, PCR product base pair sizing, and genotyping are as follows:

- The AmpFℓSTR Control DNA 9947A provides a positive control for the efficiency of the amplification step and STR genotyping using the AmpFℓSTR Identifiler Allelic Ladder.
- GeneScan-500 LIZ Size Standard is used for obtaining base pair sizing results. The GeneScan-500 LIZ Size Standard is designed for sizing DNA fragments in the 35–500 bp range, and contains 16 single-stranded fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250 (not assigned when used on the ABI PRISM 310 Genetic Analyzer), 300, 340, 350, 400, 450, 490, and 500 bases. This standard has been evaluated as an internal lane size standard and yields extremely precise sizing results of AmpFℓSTR Identifiler kit PCR products.
- The AmpFℓSTR Identifiler Allelic Ladder was developed by Applied Biosystems for accurate characterization of the alleles amplified by the AmpFℓSTR Identifiler kit. The AmpFℓSTR Identifiler Allelic Ladder contains the majority of alleles reported for the 15 loci.

Off-Scale Data

Overview

If too much sample DNA is added to the PCR reaction mixtures, the fluorescence intensity from the PCR products may exceed the linear dynamic range for detection by the instrument. This is referred to as “off-scale” data. Multicomponent analysis cannot be performed accurately on data that is off-scale. Samples with off-scale peaks will exhibit raised baselines and/or excessive “pull-up” of one or more colors under the off-scale peaks.

Analyzed data from off-scale peaks should not be used for quantitative comparisons. For example, the stutter peak that corresponds to an off-scale main peak is likely to be overestimated.

Off-Scale Data on the ABI PRISM 310

To determine if data is off-scale on the ABI PRISM® 310 Genetic Analyzer:

Step	Action
1	In the GeneScan Analysis Software, highlight the sample file row for the questionable sample in the **Analysis Control** window. Alternatively, under **View**, select **Show Offscale Regions** to highlight off-scale data with a red bar. The width of the red bar corresponds to the amount of data that is off-scale.
2	Under the **Sample** menu, choose **Raw Data.**
3	Examine the fluorescence intensity for the raw data peaks. Any peaks that are greater than 8191 relative fluorescence units (RFU) are off-scale.
4	Re-amplify the sample, if necessary. **Note** DNA samples with off-scale data should be diluted and re-amplified.

Shutting Down the Instrument

Ending the Run

If the instrument is not going to be in use for 3 or more consecutive days, it is recommended that the instrument be cleaned and shut down.

To shut down the instrument:

Step	Action
1	Remove and clean the syringe and block as previously described.
2	Discard unused polymer in the proper waste container. **Note** Do not put unused polymer back into the bottle. Polymer in the syringe decomposes over time at room temperature.
3	In the **Manual Control** window, select **Autosampler Home X, Y Axis** and click **Execute**.
4	Select **Autosampler Home Z Axis** and click **Execute**.
5	Turn off the instrument.

Dedicated Equipment and Supplies

Equipment Required

The following are equipment and supplies necessary or recommended for running AmpFℓSTR Identifiler kit data on the ABI PRISM 310 Genetic Analyzer.

Note Amplified DNA, equipment, and supplies used to handle amplified DNA should not be taken out of the amplified DNA work area. Samples that have not yet been amplified should never come into contact with these supplies and equipment.

Equipment Required:

Equipment
ABI PRISM 310 Genetic Analyzer
ABI PRISM 310 Genetic Analyzer Accessories: ♦ ABI PRISM 310 Genetic Analyzer Capillary, L_t = 47 cm, L_d = 36 cm, i.d. = 50 µm (P/N 402839), labeled with a green mark ♦ ABI PRISM 310 Genetic Analyzer Vials, 4.0 mL (P/N 401955) ♦ ABI PRISM 310 Genetic Analyzer 0.5-mL Sample Tubes (P/N 401957) ♦ ABI PRISM 310 Genetic Analyzer Septa for 0.5-mL Sample Tubes (P/N 401956) ♦ Syringe, Kloehn 1.0-mL (P/N 4304471)
Benchkote absorbent protector sheets
Flush-cutting wire cutter (P/N T-6157)
Freezer, –15 to –25 °C, non-frost-free
Gloves, disposable, powder-free
Glassware
Ice bucket
Kim Wipes
Lab coat
Microcentrifuge tubes, 1.5-mL
Microtube racks
Nalgene filter apparatus, 150–mL, 0.2-µm CN filter
Permanent ink pen
Pipette bulb
Pipettes, serological

Equipment Required: *(continued)*

Equipment
Pipette tips, sterile, disposable hydrophobic filter-plugged
Pipettors, adjustable, 1–10 µL, 2–20 µL, 20–200 µL, and 200–1000 µL
Refrigerator
Repeat pipettor and Combitips that dispense 25–125 µL (optional)
Sink
Syringe, 35 cc (optional)
Tape
Thermal cycler
Tube, 50 mL Falcon
Tube decapper, autoclavable

Reagents Required

Reagents Required:

Reagents
ABI PRISM 310 10X Genetic Analyzer Buffer with EDTA (P/N 402824)
AG501 X8 ion exchange resin (Bio-Rad)
Deionized water, PCR grade
Hi-Di™ Formamide (P/N 4311320)
GeneScan™-500 LIZ™ Size Standard (P/N 4322682)
Matrix Standard Set DS-33 [6-FAM, VIC, NED, PET, LIZ] for use with the 310/377 system (P/N 4318159)
Performance Optimized Polymer 4 (POP-4), (P/N 402838)

Software and User Documentation Required

Software and User Documentation:

Software and User Documentation
ABI PRISM 310 Collection Software, version 2.1 or higher
ABI PRISM 310 Genetic Analyzer User's Manual (P/N 903565)
ABI PRISM 310 Module GS STR POP4 (1 mL) G5
GeneScan Software v3.1 or higher

Experiments and Results

4

Overview

About This Chapter

This chapter describes various experiments performed and results obtained using the AmpFℓSTR® Identifiler™ PCR Amplification Kit.

In This Chapter

This chapter contains the following topics:

Topic	See Page
Overview	4-1
Experiments Performed Using the AmpFℓSTR Identifiler PCR Amplification Kit	4-2
Developmental Validation	4-3
Accuracy, Precision, and Reproducibility	4-6
Extra Peaks in the Electropherogram	4-19
Characterization of Loci	4-28
Species Specificity	4-30
Sensitivity	4-32
Stability	4-34
Mixture Studies	4-39
Data Interpretation	4-43
Population Data	4-44
Mutation Rate	4-55
Probability of Identity	4-56
Probability of Paternity Exclusion	4-57

Experiments Performed Using the AmpFℓSTR Identifiler PCR Amplification Kit

Importance of Validation

Validation of a DNA typing procedure for human identification applications is an evaluation of the procedure's efficiency, reliability, and performance characteristics. By challenging the procedure with samples commonly encountered in forensic and parentage laboratories, the validation process uncovers attributes and limitations, which are critical for sound data interpretation in casework (Sparkes, Kimpton, Gilbard, *et al.*, 1996; Sparkes, Kimpton, *et al.*, 1996; Wallin *et al.*, 1998).

Experiments

Experiments to evaluate the performance of AmpFℓSTR Identifiler PCR Amplification Kit were performed at Applied Biosystems. Some of these experiments were performed according to the DNA Advisory Board (DAB) Quality Assurance Standards, effective October 1, 1998 (DNA Advisory Board, 1998). The DNA Advisory Board issued quality assurance standards for forensic DNA testing laboratories.

These DAB standards describe the quality assurance requirements that a laboratory should follow to ensure the quality and integrity of the data and competency of the laboratory. DAB defines a laboratory as a facility in which forensic DNA testing is performed.

Based on these standards, Applied Biosystems has conducted experiments which comply with Standards 8.1.1 and 8.1.2 and its associated subsections. Whereas this DNA methodology is not novel, Standard 8.1.2 and its related subsections have been addressed (Holt *et al.*, 2001 and Wallin *et al.*, 2001). This chapter will discuss many of the experiments performed by Applied Biosystems and examples of results obtained. Conditions were chosen which produced maximum PCR product yield and a window in which reproducible performance characteristics were met. These experiments while not exhaustive are appropriate for a manufacturer, in our opinion. Each laboratory using the AmpFℓSTR Identifiler PCR Amplification kit should perform appropriate validation studies.

Developmental Validation

8.1.1 Developmental Validation

"*Developmental validation that is conducted shall be appropriately documented." (*DNA Advisory Board, 1998).

Critical reagent concentrations and reaction conditions (*e.g.*, thermal cycling parameters, AmpliTaq Gold® DNA polymerase activation, cycle number) to produce reliable, locus-specific amplification and appropriate sensitivity have been determined.

PCR Components

The concentration of each component of the AmpFℓSTR Identifiler kit was examined. The PCR components are Tris-HCl (pH 8.3), KCl, dNTPs, primers, AmpliTaq Gold DNA Polymerase, $MgCl_2$, bovine serum albumin, and sodium azide. The concentration for a particular component was established to be in the window that meets the reproducible performance characteristics of specificity and sensitivity.

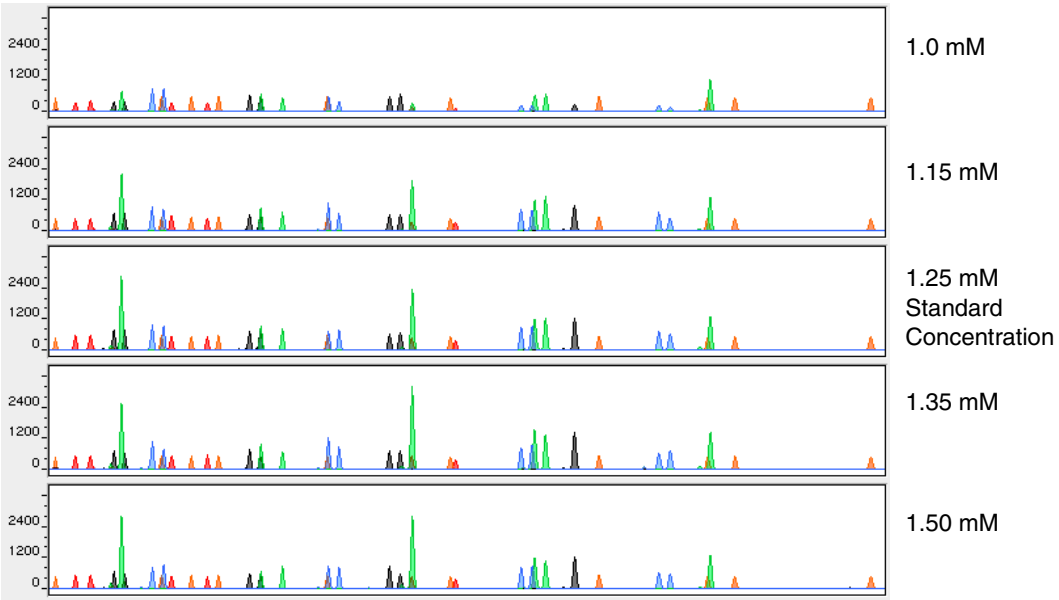


Figure 4-1 A 1 ng amplification of genomic DNA varying the magnesium chloride concentration, analyzed on the ABI PRISM® 310 Genetic Analyzer

Thermal Cycler Parameters

Thermal cycling parameters were established for amplification of the AmpFℓSTR Identifiler kit in the GeneAmp® PCR Systems 9600 and 9700. Thermal cycling times and temperatures of GeneAmp PCR systems were verified. Annealing and denaturation temperature windows were tested around each stipend to verify that a ±1.5 °C window produced a specific PCR product with the desired sensitivity of at least 1 ng of AmpFℓSTR® Control DNA 9947A.

The effects of denaturation and annealing temperatures on the amplification of AmpFℓSTR Identifiler kit loci were examined using AmpFℓSTR Control DNA 9947A and two DNA samples.

The denaturation temperatures tested were 92.5, 94, and 95.5 °C, all for 1-minute hold times on the GeneAmp PCR System 9700. The annealing temperatures tested were 55, 57, 59, 61, and 63 °C (see Figure 4-2), also for 1-minute hold times in the GeneAmp PCR System 9700. The PCR products were analyzed using the ABI PRISM 310 Genetic Analyzer.

Neither preferential nor differential amplification was observed in the denaturation temperature experiments. Of the tested annealing temperatures, 55, 57, 59, and 61 °C produced robust profiles. At 63 °C, the yield of the majority of loci was significantly reduced. This should pose no problem with routine thermal cycler calibration and when following the recommended amplification protocol. Preferential amplification was not observed at any of the tested annealing temperatures.

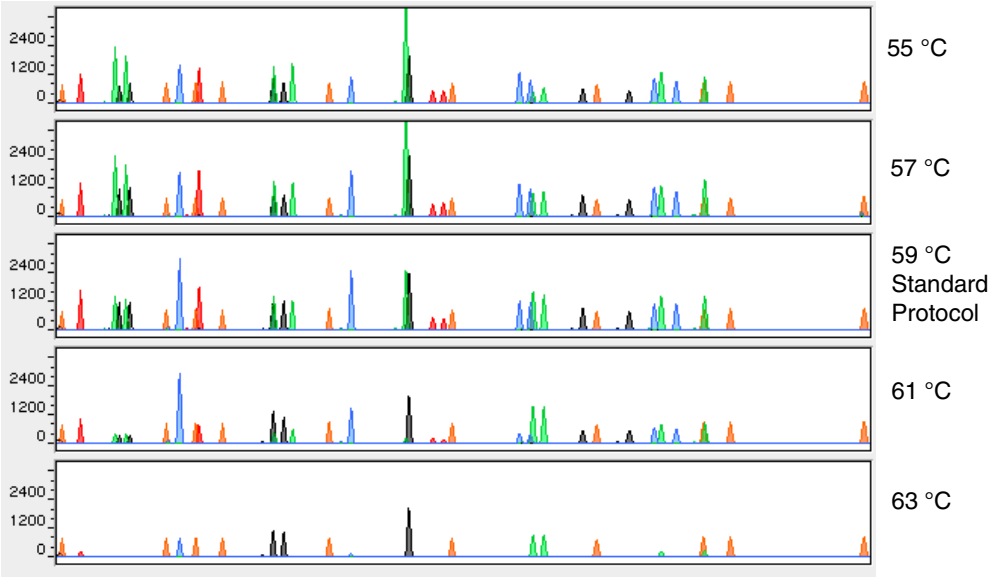


Figure 4-2 An amplification of 1 ng of genomic DNA, amplified while varying the annealing temperature, analyzed on the ABI PRISM 310 Genetic Analyzer

AmpliTaq Gold DNA Polymerase Activation

The thermal cycler program for the AmpFℓSTR Identifiler kit includes an initial incubation to allow for AmpliTaq Gold DNA polymerase activation. Polymerase activation times and temperatures were tested in the GeneAmp PCR Systems 9600 and 9700.

Five activation times (5, 8, 11, 14 and 17 minutes) were tested previously for the AmpFℓSTR kits (Wallin *et al.*, 1998). A plateau in relative fluorescent signal (RFU) of the AmpFℓSTR Identifiler kit loci was reached at approximately 8 minutes and was maintained through the 17–minute time point. The 11–minute activation time was determined to be optimal based on this plateau (within a ± >25% window).

PCR Cycle Number

AmpFℓSTR Identifiler kit reactions were amplified for 27, 28, 29, 30, and 31 cycles on the GeneAmp PCR System 9700 using 1.0 ng of three DNA samples. As expected, PCR product increased with the number of cycles. A full profile was generated at 27 cycles; off-scale data were collected for several allele peaks at 31 cycles.

While none of the cycle numbers tested produced nonspecific peaks, 28 cycles was found to give optimal sensitivity when the amplified products were examined on ABI PRISM 310 Genetic Analyzers. Additionally, the cycle number was set to avoid detection of low quantities of DNA (20 pg or less). At 28 cycles, 1.0 ng of AmpFℓSTR Control DNA 9947A amplifies reliably and specifically following the conditions outlined in this user manual.

8.1.2 Accuracy

"Novel forensic DNA methodologies shall undergo developmental validation to ensure the accuracy, precision and reproducibility of the procedure." (DAB, 1998).

Laser-induced fluorescence detection systems of length polymorphism at short tandem repeat loci is not a novel methodology (Holt *et al.*, 2001 and Wallin *et al.*, 2001). However, accuracy and reproducibility of AmpFℓSTR Identifiler kit profiles have been determined from various sample types.

Figure 4-3 illustrates the size differences that are typically observed between sample alleles and allelic ladder alleles on the ABI PRISM 310 Genetic Analyzer with POP-4™ polymer. The x-axis in Figure 4-5 represents the nominal base pair sizes for the AmpFℓSTR Identifiler Allelic Ladder, and the dashed lines parallel to the x-axis represent the ±0.5-bp windows. The y-axis is the deviation of each sample allele size from the corresponding allelic ladder allele size. The data include a total of 2269 alleles from 70 population database samples. All sample alleles are within 0.5 bp of a corresponding allele in an allelic ladder.

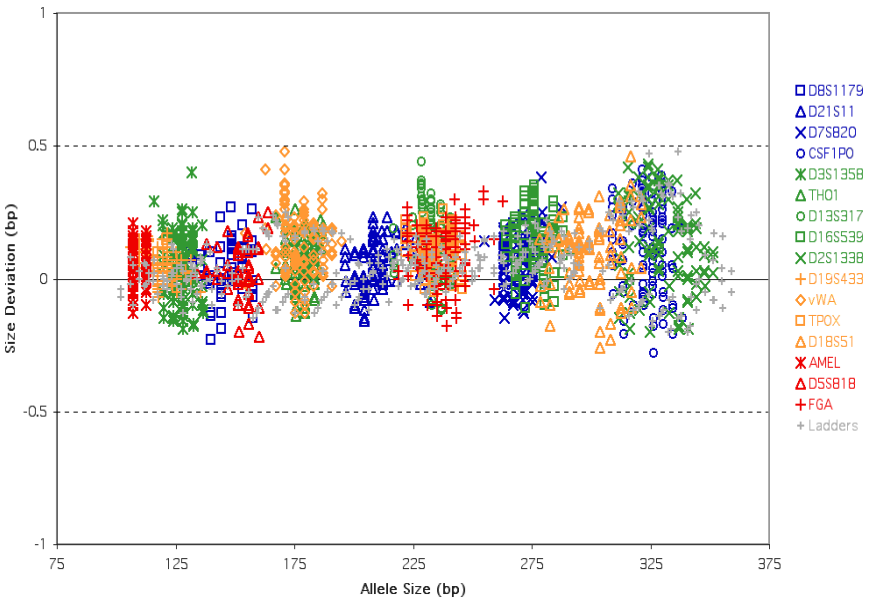


Figure 4-3 Size deviation of 70 samples and two allelic ladders from one injection of allelic ladder on a single ABI PRISM 310 Genetic Analyzer run

Precision and Size Windows

Sizing precision allows for determining accurate and reliable genotypes. Sizing precision was measured on the ABI PRISM 310 Genetic Analyzer. As indicated in the Automated Genotyping section, the recommended method for genotyping is to employ a ±0.5-bp "window" around the size obtained for each allele in the AmpFℓSTR Identifiler Allelic Ladder. A ±0.5-bp window allows for the detection and correct assignment of alleles. An allele that sizes only one base pair different from an allele in the allelic ladder will not be incorrectly typed and will be identified as off-ladder. Any sample allele that sizes outside a window could be either of the following:

- an "off-ladder" allele, *i.e.*, an allele of a size that is not represented in the AmpFℓSTR Identifiler Allelic Ladder
- an allele that does correspond to an allelic ladder allele, but whose size is just outside a window because of measurement error

The measurement error inherent in any sizing method can be defined by the degree of precision in sizing an allele multiple times. Precision is measured by calculating the standard deviation in the size values obtained for an allele that is run in several lanes of one gel or in several injections in one capillary run.

Table 4-1 on page 4-10 indicates typical precision results obtained from the seven injections of the AmpFℓSTR Identifiler Allelic Ladder analyzed on the ABI PRISM 310 Genetic Analyzer (47-cm capillary and POP-4 polymer). The internal lane size standard used was GeneScan-500 LIZ. These results were obtained within a set of injections on a single capillary.

As indicated above, sample alleles may occasionally size outside of the ±0.5-bp window for a respective allelic ladder allele because of measurement error. The frequency of such an occurrence is lowest in detection systems having the smallest standard deviations in sizing. Figure 4-3 on page 4-6 illustrates the tight clustering of allele sizes obtained on the ABI PRISM 310 Genetic Analyzer, where the standard deviation in sizing is typically less than 0.15 bp. The instance of a sample allele sizing outside of the ±0.5-bp window because of measurement error is relatively rare when the standard deviation in sizing is approximately 0.15 bp or less (Smith, 1995).

For sample alleles that do not size within a ±0.5-bp window, the PCR product must be rerun to distinguish between a true off-ladder allele *vs.* measurement error of a sample allele that corresponds with an allele in the allelic ladder. Repeat analysis, when necessary, provides an added level of confidence to the final allele assignment. Genotyper® software automatically flags sample alleles that do not size within the prescribed window around an allelic ladder allele.

It is important to note that while the precision within a gel or set of capillary injections is very good, the determined allele sizes vary between platforms. Cross-platform sizing differences arise from a number of parameters, including type and concentration of polymer mixture, run temperature, and electrophoresis conditions. Variations in sizing can also be found between runs on the same instrument and between runs on different instruments because of these parameters. We strongly recommend that the allele sizes obtained be compared to the sizes obtained for known alleles in the AmpFℓSTR Identifiler Allelic Ladder from the same run and then converted to genotypes (as described in the Automated Genotyping section) For more information on precision and genotyping, see Lazaruk *et al.*, 1998 and Mansfield *et al.*,1998.

1187 population database DNA samples have been typed using the AmpFℓSTR Identifiler PCR Amplification Kit(see “About the Primers” on page 1-2). These samples have been previously genotyped with concordant results of the same loci, using other AmpFℓSTR kits.

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder:

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
Amelogenin		
X	107.02	0.04
Y	112.61	0.02
CSF1PO		
6	304.69	0.08
7	309.01	0.10
8	313.30	0.10
9	317.55	0.11
10	321.97	0.12
11	325.86	0.11
12	329.97	0.13
13	334.00	0.10
14	338.04	0.11
15	341.84	0.08
D2S1338		
15	307.30	0.11
16	311.65	0.11
17	315.91	0.12
18	320.16	0.12
19	324.34	0.12
20	328.44	0.08
21	332.58	0.11
22	336.62	0.09
23	340.57	0.11
24	344.18	0.07
25	347.78	0.07
26	351.39	0.07
27	355.08	0.07
28	358.77	0.05

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder: *(continued)*

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
D3S1358		
12	111.96	0.06
13	116.04	0.04
14	119.99	0.04
15	123.89	0.02
16	128.06	0.05
17	132.24	0.05
18	136.30	0.06
19	140.43	0.03
D5S818		
7	134.14	0.05
8	138.21	0.04
9	142.56	0.04
10	147.02	0.06
11	151.31	0.01
12	155.63	0.05
13	159.81	0.06
14	164.04	0.07
15	167.95	0.05
16	172.09	0.05
D7S820		
6	255.15	0.08
7	259.21	0.07
8	263.24	0.07
9	267.26	0.09
10	271.32	0.08
11	275.35	0.06
12	279.42	0.07
13	283.42	0.06
14	287.48	0.10
15	291.58	0.06

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder: *(continued)*

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
D8S1179		
8	123.29	0.07
9	127.32	0.05
10	131.41	0.05
11	135.49	0.04
12	139.73	0.04
13	144.25	0.03
14	148.71	0.06
15	153.16	0.07
16	157.51	0.07
17	161.72	0.05
18	165.84	0.07
19	169.92	0.05
D13S317		
8	216.87	0.05
9	220.83	0.05
10	224.77	0.07
11	228.88	0.07
12	232.81	0.05
13	236.68	0.07
14	240.69	0.06
15	244.68	0.09
D16S539		
5	252.37	0.08
8	264.30	0.07
9	268.32	0.08
10	272.32	0.06
11	276.37	0.07
12	280.37	0.09
13	284.34	0.07
14	288.44	0.09
15	292.51	0.07

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder: *(continued)*

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
D18S51		
7	262.07	0.08
9	270.22	0.06
10	274.34	0.09
10.2	276.36	0.06
11	278.41	0.08
12	282.49	0.05
13	286.57	0.06
13.2	288.63	0.05
14	290.77	0.04
14.2	292.78	0.05
15	294.91	0.07
16	299.07	0.06
17	303.50	0.07
18	307.94	0.09
19	312.40	0.11
20	316.71	0.09
21	320.99	0.14
22	325.24	0.11
23	329.40	0.11
24	333.54	0.15
25	337.67	0.11
26	341.56	0.09
27	345.24	0.08

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder: *(continued)*

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
D19S433		
9	101.99	0.05
10	105.88	0.05
11	109.78	0.04
12	113.64	0.02
12.2	115.61	0.02
13	117.56	0.03
13.2	119.55	0.02
14	121.46	0.03
14.2	123.47	0.02
15	125.45	0.05
15.2	127.43	0.05
16	129.44	0.05
16.2	131.46	0.05
17	133.42	0.03
17.2	135.44	0.06

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder: *(continued)*

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
D21S11		
24	184.86	0.04
24.2	186.82	0.02
25	188.77	0.03
26	192.69	0.05
27	196.56	0.04
28	200.41	0.05
28.2	202.36	0.05
29	204.32	0.03
29.2	206.31	0.02
30	208.29	0.07
30.2	210.24	0.05
31	212.23	0.05
31.2	214.14	0.06
32	216.14	0.04
32.2	218.10	0.04
33	220.14	0.05
33.2	222.07	0.04
34	224.10	0.07
34.2	226.02	0.06
35	228.07	0.06
35.2	230.01	0.07
36	232.04	0.07
37	236.00	0.03
38	239.94	0.08

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder: *(continued)*

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
FGA		
17	214.81	0.07
18	218.80	0.06
19	222.79	0.07
20	226.81	0.06
21	230.76	0.08
22	234.78	0.07
23	238.81	0.05
24	242.83	0.07
25	246.88	0.06
26	250.96	0.06
26.2	253.00	0.09
27	254.97	0.08
28	259.02	0.10
29	263.12	0.08
30	267.26	0.09
30.2	269.07	0.10
31.2	273.17	0.09
32.2	277.24	0.08
33.2	281.33	0.09
42.2	319.83	0.14
43.2	324.04	0.14
44.2	328.26	0.13
45.2	332.42	0.16
46.2	336.43	0.14
47.2	340.42	0.14
48.2	344.15	0.10
50.2	351.45	0.05
51.2	355.13	0.05

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder: *(continued)*

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
TH01		
4	163.29	0.04
5	167.36	0.03
6	171.40	0.05
7	175.40	0.03
8	179.38	0.04
9	183.36	0.05
9.3	186.93	0.02
10	187.29	0.04
11	191.23	0.03
13.3	201.94	0.05
TPOX		
6	222.07	0.04
7	226.02	0.06
8	229.91	0.03
9	233.86	0.06
10	237.88	0.07
11	241.83	0.06
12	245.77	0.07
13	249.78	0.08

Table 4-1 Example of precision results of seven injections of the AmpFℓSTR Identifiler Allelic Ladder: *(continued)*

	ABI PRISM 310 Genetic Analyzer	
Allele	**Mean**	**S.D.**
vWA		
11	154.59	0.08
12	158.87	0.07
13	163.00	0.05
14	167.27	0.05
15	171.15	0.05
16	175.15	0.04
17	179.15	0.04
18	183.08	0.04
19	187.00	0.04
20	190.93	0.05
21	194.80	0.05
22	198.62	0.06
23	202.44	0.05
24	206.69	0.08

Extra Peaks in the Electropherogram

Causes of Extra Peaks

To further demonstrate reproducibility, 1187 population database DNA samples have been typed using the AmpFℓSTR Identifiler PCR Amplification Kit. These samples have been previously genotyped with concordant results of the same loci using other AmpFℓSTR kits.

Peaks other than the target alleles may be detected on the electropherogram displays. Several causes for the appearance of extra peaks, including the stutter product (found at the n–4 position), incomplete 3´ A nucleotide addition (found at the n–1 position), artifacts and mixed DNA samples (see 8.1.2.2).

Stutter Products

The PCR amplification of tetranucleotide STR loci typically produces a minor product peak four bases shorter (n–4) than the corresponding main allele peak. This is referred to as the stutter peak or product. Sequence analysis of stutter products at tetranucleotide STR loci has revealed that the stutter product is missing a single tetranucleotide core repeat unit relative to the main allele (Walsh *et al.*,1996).

The proportion of the stutter product relative to the main allele (percent stutter) is measured by dividing the height of the stutter peak by the height of the main allele peak. Such measurements have been made for amplified samples at the loci used in the AmpFℓSTR Identifiler kit. All data were generated on the ABI PRISM 310 Genetic Analyzer.

Some of the general conclusions from these measurements and observations are as follows:

- For each AmpFℓSTR Identifiler kit locus, the percent stutter generally increases with allele length, as shown in Figures 4-4, 4-5, 4-7 and 4-8. Smaller alleles display a lower level of stutter relative to the longer alleles within each locus. This is reflected in Figures 4-4 through 4-7, where minimal data points are plotted for some smaller alleles, as stutter could not be detected for many of these samples.
- For the alleles within a particular locus, the percent stutter is generally greater for the longer allele in a heterozygous sample (this is related to the first point above).
- Each allele within a locus displays percent stutter that is reproducible.
- The highest percent stutter observed for each allele is as follows: CSF1PO, 9.2%; D2S1338, 11.1%; D3S1358, 10.7%; D5S818, 6.8%; D7S820, 8.2%; D8S1179, 8.2%; D13S317, 8.0%; D16S539,

10.4%; D18S51, 17.0%; D19S433, 13.3%; D21S11, 9.4%; FGA, 14.7%; TH01, 5.1%; TPOX, 4.8% and vWA, 12.6%.

- The highest observed percent stutter for each locus is included as the filtering step in Genotyper software. Peaks in the stutter position that are above the highest observed percent stutter will not be filtered. Peaks in the stutter position that have not been filtered and remain labeled can be further evaluated. For evaluation of mixed samples, see “Mixed Samples.”
- The percent stutter does not change significantly with the recommended quantity of input DNA, for on-scale data. The measurement of percent stutter may be unusually high for main peaks that are off-scale.
- The percent stutter for allele 15 in D3S1358 (Figure 4-5) is artificially increased due to a reproducible artifact (Figure 4-8) observed in the green dye lanes at this position. When analyzing samples which contain a D3S1358 allele 15, we recommend careful examination due to the contribution that this identified artifact may add to the observed peak height or area. The highest percent stutter for D3S1358 is not inconclusive of allele 15.

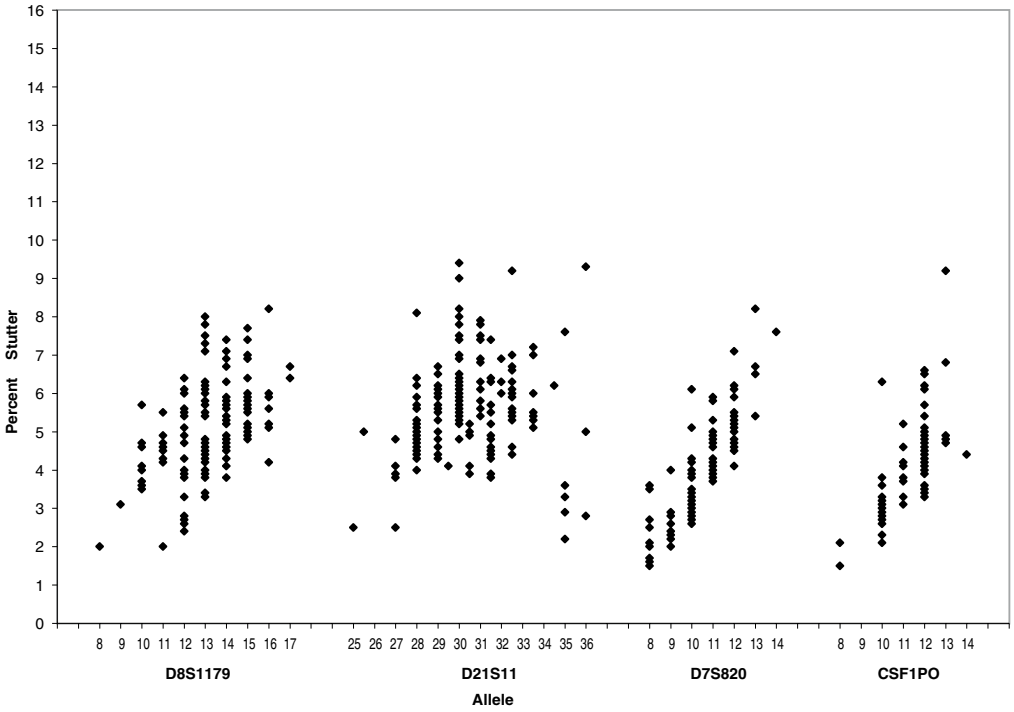


Figure 4-4 Stutter percentages for the D8S1179, D21S11, D7S820, and CSF1PO loci

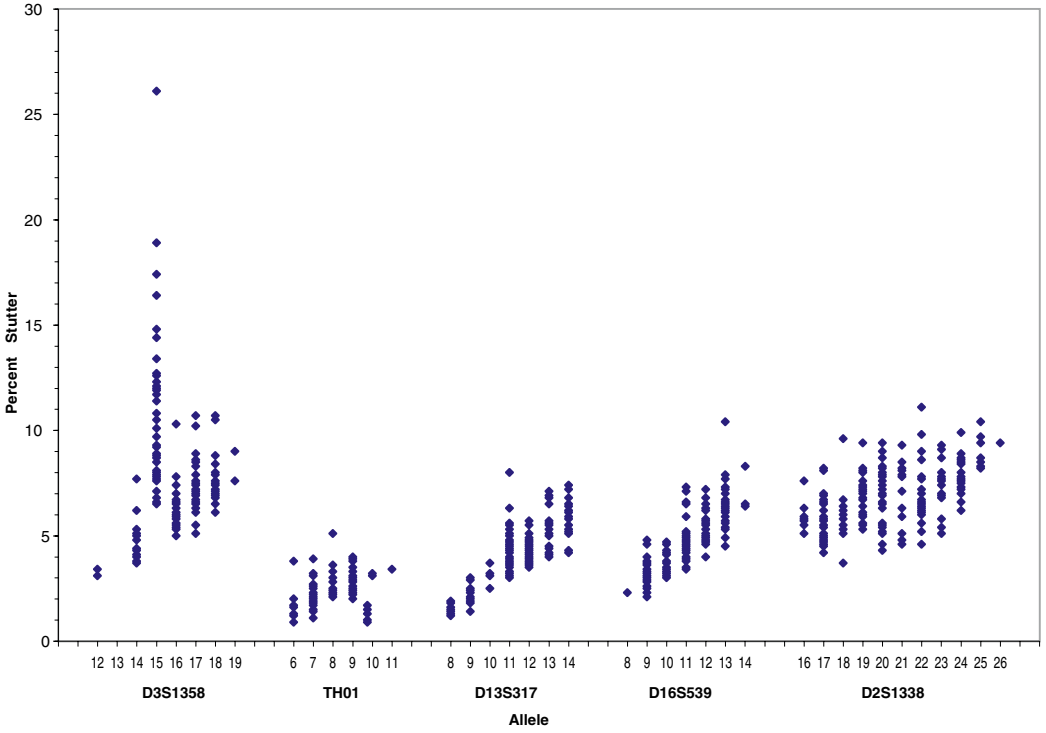


Figure 4-5 Stutter percentages for the D3S1358, TH01, D13S317, D16S539, and D2S1338 loci. See the comment on page 4-17 regarding stutter at allele 15 of D3S1358

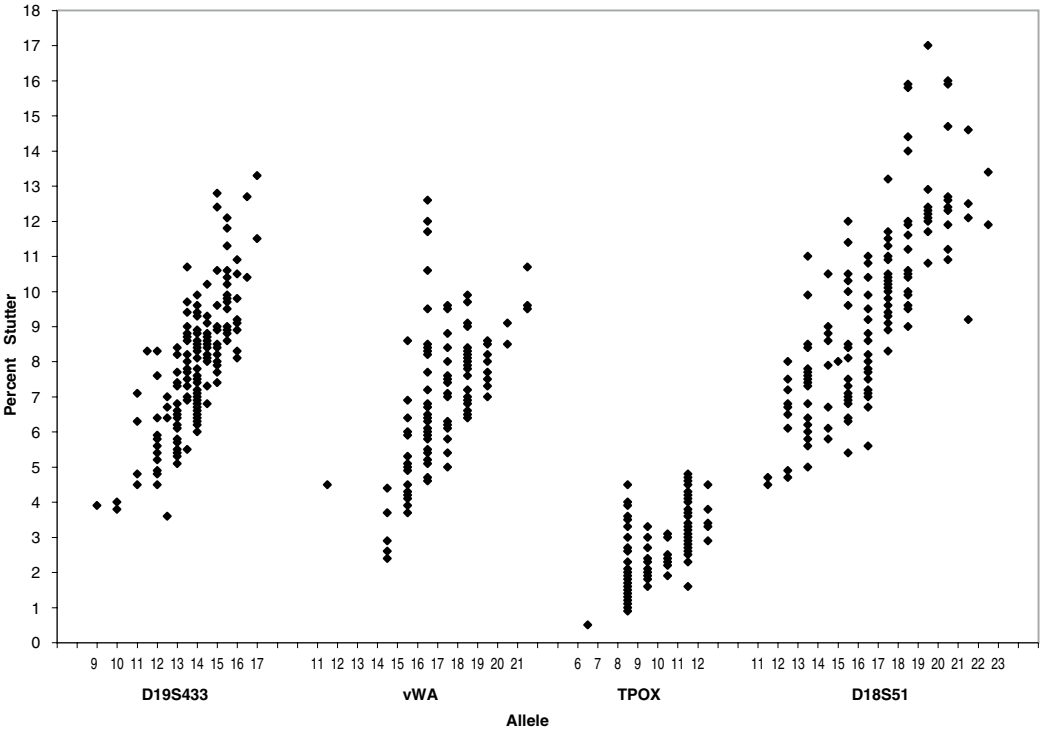


Figure 4-6 Stutter percentages for the D19S433, vWA, TPOX, and D18S51 loci

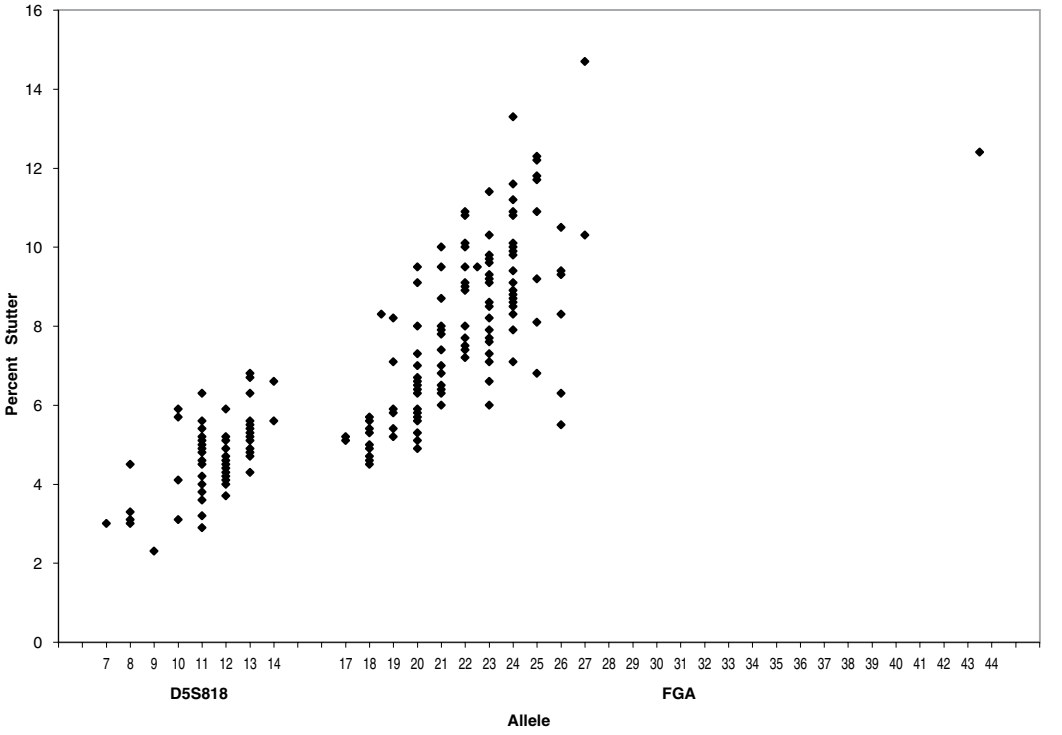


Figure 4-7 Stutter percentages for the D5S818 and FGA loci

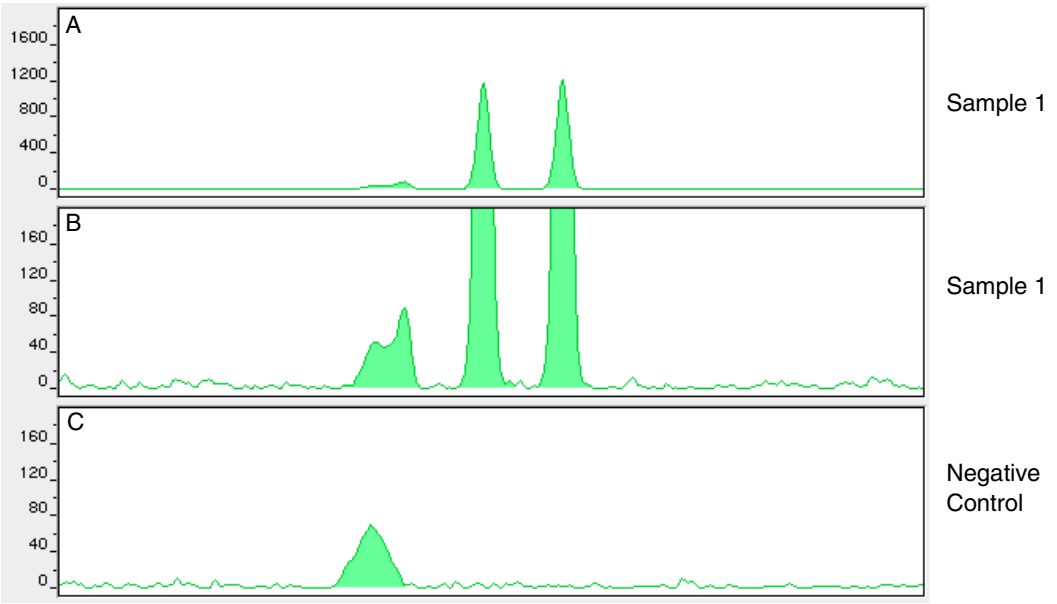


Figure 4-8 Sample 1 in panel A and panel B has a profile of 15, 16 for D3S1358. The amount of stutter can not be accurately measured due to the VIC™ dye artifact. Note the degree of magnification (y-axis) used in panels B and C to illustrate the artifact. Data was produced on the ABI PRISM 310 Genetic Analyzer.

Addition of 3´ A Nucleotide

AmpliTaq Gold® enzyme, like many other DNA polymerases, can catalyze the addition of a single nucleotide (predominately adenosine) to the 3´ ends of double-stranded PCR products (Clark, 1988; Magnuson *et al.*,1996). This non-template addition results in a PCR product that is one base pair longer than the actual target sequence, and the PCR product with the extra nucleotide is referred to as the "+A" form.

The efficiency of "A addition" is related to the particular sequence of the DNA at the 3´ end of the PCR product. The AmpFℓSTR Identifiler kit includes two main design features that promote maximum A addition:

- The primer sequences have been optimized to encourage A addition.
- The final extension step is 60 °C for 60 min.

This final extension step gives the AmpliTaq Gold DNA Polymerase extra time to complete A addition to all double-stranded PCR product. STR systems that have not been optimized for maximum A addition may have "split peaks", where each allele is represented by two peaks one base pair apart.

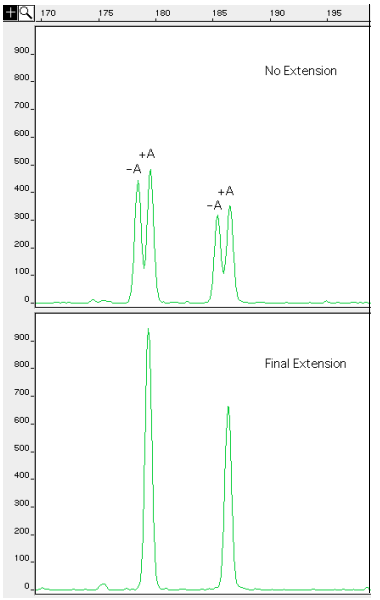


Figure 4-9 Split peaks resulting from incomplete A nucleotide addition due to omission of the 60-minute extension step

The AmpliTaq Gold DNA Polymerase generally requires extra time to complete the A nucleotide addition at the 3´ end of the PCR products.

Lack of full A nucleotide addition may be observed in AmpFℓSTR Identifiler kit results when the amount of input DNA is greater than recommended protocols. The reason for this is that more time is needed for AmpliTaq Gold DNA Polymerase to add the A nucleotide to all molecules as more PCR product is generated. Amplification of too much input DNA will also result in off-scale data.

Artifacts

Artifacts, or anomalies, have been seen in data produced on the ABI PRISM® 310 Genetic Analyzer when using the AmpFℓSTR Identifiler kit. The shape of these artifacts is not consistent with the shape of labeled DNA fragments as seen on the ABI PRISM 310 Genetic Analyzer. Artifacts may or not be reproducible.

Figure 4-10 on page 4-27 demonstrates reproducible artifacts while using the AmpFℓSTR Identifiler kit. A user of the AmpFℓSTR Identifiler kit on the ABI PRISM 310 Genetic Analyzer should consider these artifacts when interpreting data.

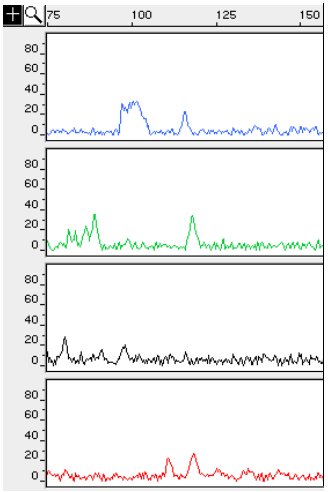


Figure 4-10 Reproducible anomalies in the blue, green, yellow, and red dye electropherograms when using the AmpFℓSTR Identifiler PCR Amplification Kit. Genotyping may result in the detection of these artifacts as off-ladder alleles, or "OL Alleles?". Note the degree of magnification (y-axis) used in this figure to illustrate these artifacts, data produced on the ABI PRISM 310 Genetic Analyzer.

Artifacts can be intermittent and are not always reproducible. In our experience, non-reproducible artifacts can be correlated to sources other than the kit (*e.g.*, spikes). An intermittent artifact is not observed in the same position upon re-injection. The Identifiler kit user should consider these artifacts when interpreting data.

Characterization of Loci

8.1.2.1 Documentation

"*Documentation exists and is available which defines and characterizes the locus.*" (DAB, 1998).

Overview

This section describes basic characteristics of the 16 loci that are amplified with the AmpFℓSTR Identifiler kit. These loci have been previously characterized.

Nature of the Polymorphisms

The primers for the Amelogenin locus flank a six-base pair deletion within intron 1 of the X homologue. Amplification results in 107-bp and 113-bp products from the X and Y chromosomes, respectively. (Sizes are the actual base pair size according to sequencing results, including 3' A nucleotide addition.) The remaining AmpFℓSTR Identifiler kit loci are all tetranucleotide short tandem repeat (STR) loci. The length differences among alleles of a particular locus result from differences in the number of 4–bp repeat units.

Some alleles in the AmpFℓSTR Identifiler Allelic Ladder containing partial repeat units in population database and nonhuman primate DNA samples have been subjected to DNA sequencing at Applied Biosystems (Lazaruk, *et al.*, 2001). In addition, other groups in the forensic community have sequenced alleles at some of these loci (Nakahori *et al.*, 1991; Puers *et al.*, 1993; Möller *et al.*, 1994; Barber *et al.*, 1995; Möller and Brinkmann, 1995; Barber *et al.*, 1996; Barber and Parkin, 1996; Brinkmann *et al.*, 1998; Momhinweg *et al.*, 1998; Watson *et al.*, 1998). Among the various sources of sequence data on the AmpFℓSTR Identifiler kit loci, there is consensus on the repeat patterns and structure of the STRs.

Inheritance

The AmpFℓSTR loci have been validated by family studies to demonstrate their mode(s) of inheritance.

The Centre d'Etude du Polymorphisme Humain (CEPH) has collected DNA from 39 families of Utah Mormon, French Venezuelan, and Amish descent. These DNA sets have been extensively studied all over the world and are routinely used to characterize the mode of inheritance of various DNA loci. Each family set contains three generations, generally including four grandparents, two parents, and several offspring. Consequently, the CEPH family DNA sets are ideal for studying inheritance patterns (Begovich *et al.*,1992).

Four CEPH family DNA sets were examined. One and a half nanograms of DNA from each sample were amplified using the AmpFℓSTR SGM Plus™ kit, followed by analysis using an

ABI PRISM® 377 DNA Sequencer. The families examined included #1331 (11 offspring), #13291 (9 offspring), #13292 (9 offspring), and #13294 (8 offspring), representing 37 meiotic divisions. The results confirmed that the loci are inherited according to Mendelian rules, as has been reported in the literature (Nakahori *et al.*,1991; Edwards *et al.*,1992; Kimpton *et al.*,1992; Mills *et al.*,1992; Sharma and Litt, 1992; Li *et al.*,1993; Straub *et al.*,1993).

Mapping

The AmpFℓSTR kit loci Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA have been mapped and the chromosomal locations have been published (Nakahori *et al.*, 1991; Edwards *et al.*,1992; Kimpton *et al.*,1992; Mills *et al.*,1992; Sharma and Litt,1992; Li *et al.*,1993; Straub *et al.*,1993; Barber and Parkin,1996).

8.1.2.2 Species Specificity

"*Species specificity, sensitivity, stability and mixture studies are conducted.*" (DAB, 1998).

The AmpFℓSTR Identifiler kit provides the required degree of specificity such that it is specific to primates. Other species do not amplify for the loci tested, with the exception of the Amelogenin locus.

Nonhuman Studies

Nonhuman DNA may be present in forensic casework samples. The AmpFℓSTR Identifiler kit provides the required degree of specificity such that it is specific to primates for the species tested (with the exception of the Amelogenin locus).The following experiments were conducted to investigate interpretation of AmpFℓSTR Identifiler kit results from nonhuman DNA sources.

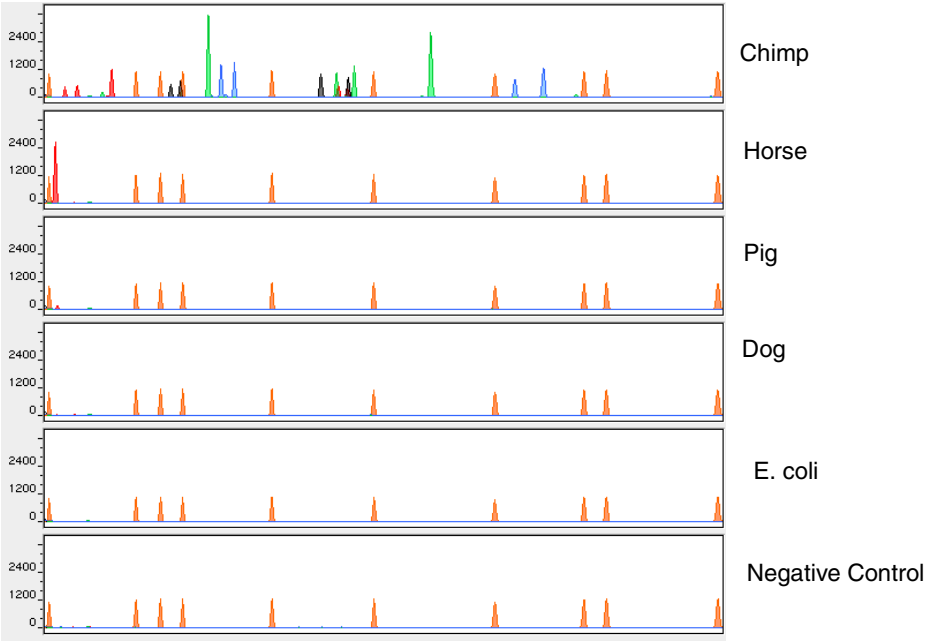


Figure 4-11 Representative electropherograms of a primate, non-primates, a microorganism, and a negative control are shown. All samples were analyzed on an ABI PRISM 310 Genetic Analyzer. The peaks dipicted in orange are the GeneScan-500 LIZ size standard

The extracted DNA samples were amplified in AmpFℓSTR Identifiler kit reactions and analyzed using the ABI PRISM 310 Genetic Analyzer.

- Primates—gorilla, chimpanzee, orangutan, and macaque (1.0 ng each).
- Non primates—mouse, dog, pig, cat, horse, chicken and cow (2.5 ng each).
- Bacteria and yeast—*Brochothrix*, *Escherichia*, *Neisseria*, *Pseudomonas*, *Bacillus*, *Staphylococcus* (approximately 5 ng each), and *Saccharomyces* (1 ng).

The primate DNA samples all amplified, producing fragments within the 100–400 base pair region (Lazaruk, *et al*., 2001; Wallin *et al*.,1998).

The microorganisms, chicken, cow, cat and mouse did not yield detectable product. Horse, dog, and pig produced a 103-bp fragment near the Amelogenin locus in PET™ dye.

Sensitivity

8.1.2.2 Sensitivity

"*Species specificity, sensitivity, stability and mixture studies are conducted.*" (DAB, 1998).

Effect of DNA Quantity on Results

Importance of Quantitation

The amount of input DNA added to the AmpFℓSTR Identifiler PCR Amplification kit should be between 0.5 and 1.25 ng. The DNA sample should be quantitated prior to amplification using a system such as the QuantiBlot® Human DNA Quantitation Kit (P/N N808-0114), see Appendix E. The final DNA concentration should be in the range of 0.05–0.125 ng/µL so that 0.5–1.25 ng of DNA will be added to the PCR reaction in a volume of 10 µL. If the sample contains degraded DNA, amplification of additional DNA may be beneficial.

If too much DNA is added to the PCR reaction, then the increased amount of PCR product that is generated can result in the following:

- Fluorescence intensity that exceeds the linear dynamic range for detection by the instrument ("off-scale" data)

  Off-scale data is a problem for two reasons:

  – Quantitation (peak height and area) for off-scale peaks is not accurate. For example, an allele peak that is off-scale can cause the corresponding stutter peak to appear higher in relative intensity, thus increasing the calculated percent stutter.

  – Multicomponent analysis of off-scale data is not accurate, which results in poor spectral separation ("pull-up").

- Incomplete A nucleotide addition

The sample can be re-amplified using less DNA.

When the total number of allele copies added to the PCR is extremely low, unbalanced amplification of the two alleles of a heterozygous individual may occur (Walsh *et al.*,1992; Wallin *et al.*,1998) due to stochastic fluctuation in the ratio of the two different alleles (Sensabaugh *et al.*,1991). The PCR cycle number and amplification conditions have been specified to produce low peak heights for a sample containing 20-pg human genomic DNA. Low peak heights should be interpreted with caution.

Individual laboratories may find it useful to determine an appropriate minimum peak height threshold based on their own results and instruments using low amounts of input DNA.

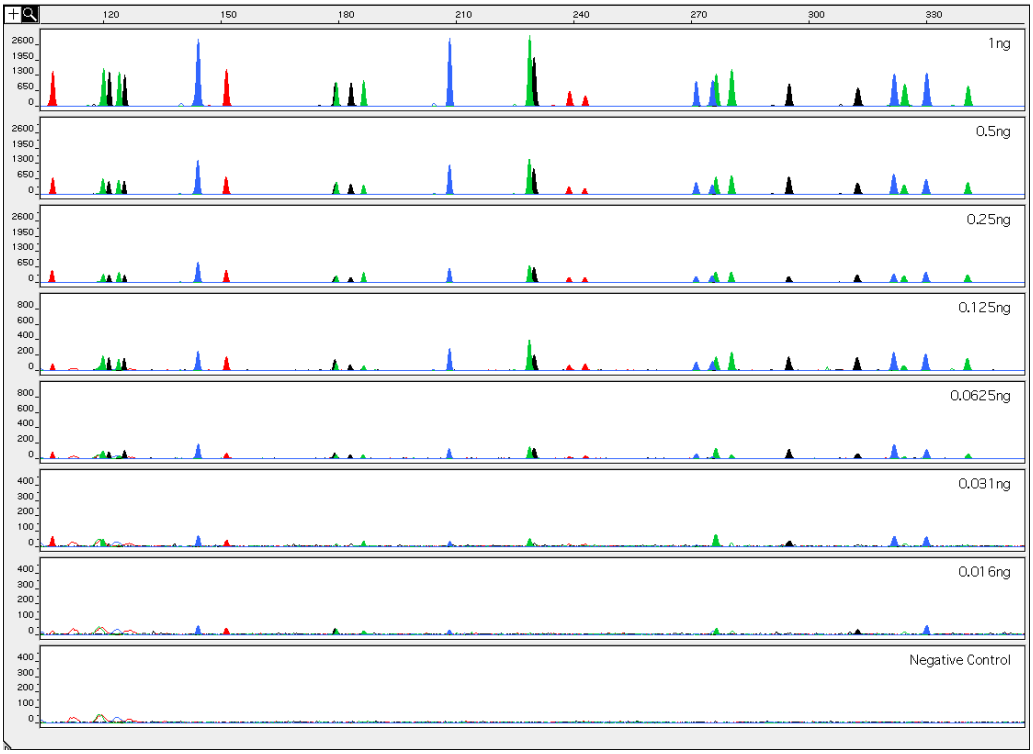


Figure 4-12 Effect of amplifying various amounts of DNA ranging from 16 pg to 1 ng. Note that the y-axis scale is magnified for the lower amounts of DNA, analyzed using the ABI PRISM 310 Genetic Analyzer

Stability

8.1.2.2 Stability

"*Species specificity, sensitivity, stability and mixture studies are conducted.*" (DAB, 1998).

Overview

Lack of Amplification of Some Loci

As with any multi-locus system, the possibility exists that not every locus will amplify. This is most often observed when the DNA sample contains PCR inhibitors or when the DNA sample has been severely degraded. Since each locus is an independent marker, whose results are not based upon information provided by the other markers, results generally can still be obtained from the loci that do amplify.

Differential and Preferential Amplification

Differential amplification can be defined as the difference in the degree of amplification of each locus within a co-amplified system, such that one or more loci may amplify to a greater extent compared to the other loci. Preferential amplification is used in this manual to describe differences in the amplification efficiency of two alleles at a single locus.

Preferential amplification of alleles in systems that distinguish alleles based on length polymorphisms is most likely to be observed when the alleles differ significantly in base pair size. Since most AmpFℓSTR Identifiler kit loci have small size ranges, the potential for preferential amplification of alleles is low.

Effect of Inhibitors

Heme compounds have been identified as PCR inhibitors in DNA samples extracted from bloodstains (DeFranchis *et al.*,1988; Akane *et al.*, 1994). It is believed that the inhibitor is co-extracted and co-purified with the DNA and subsequently interferes with PCR by inhibiting polymerase activity.

Bovine serum albumin (BSA) can prevent or minimize the inhibition of PCR, most likely by binding to the inhibitor (Comey *et al.*, 1994). Since the presence of BSA can improve the amplification of DNA from blood-containing samples, BSA has been included in the AmpFℓSTR® PCR Reaction Mix at 4 μg per 25-μL amplification. BSA has also been identified as an aid in overcoming inhibition from samples containing dyes, such as in denim (Comey *et al.*, 1994).

To examine the effects of hematin on the amplification results obtained by the AmpFℓSTR Identifiler kit, DNA samples were amplified using the AmpFℓSTR Identifiler kit reagents (including the BSA-containing PCR reaction mix) in the presence of varying concentrations of purified hematin. The concentrations of hematin used were 0 μM,10 μM, 12 μM, 14 μM, 16 μM, 18 μM and 20 μM. When the amount of hematin was

increased to a concentration that started to inhibit the PCR, CSF1PO and D2S1338 were the first loci to exhibit decreased amplification, followed by D16S539 and D18S51.

Differential amplification was observed in the presence of increasing amounts of hematin. Moreover, as the concentration of hematin was increased, the overall yield of products was reduced particularly for the larger loci.

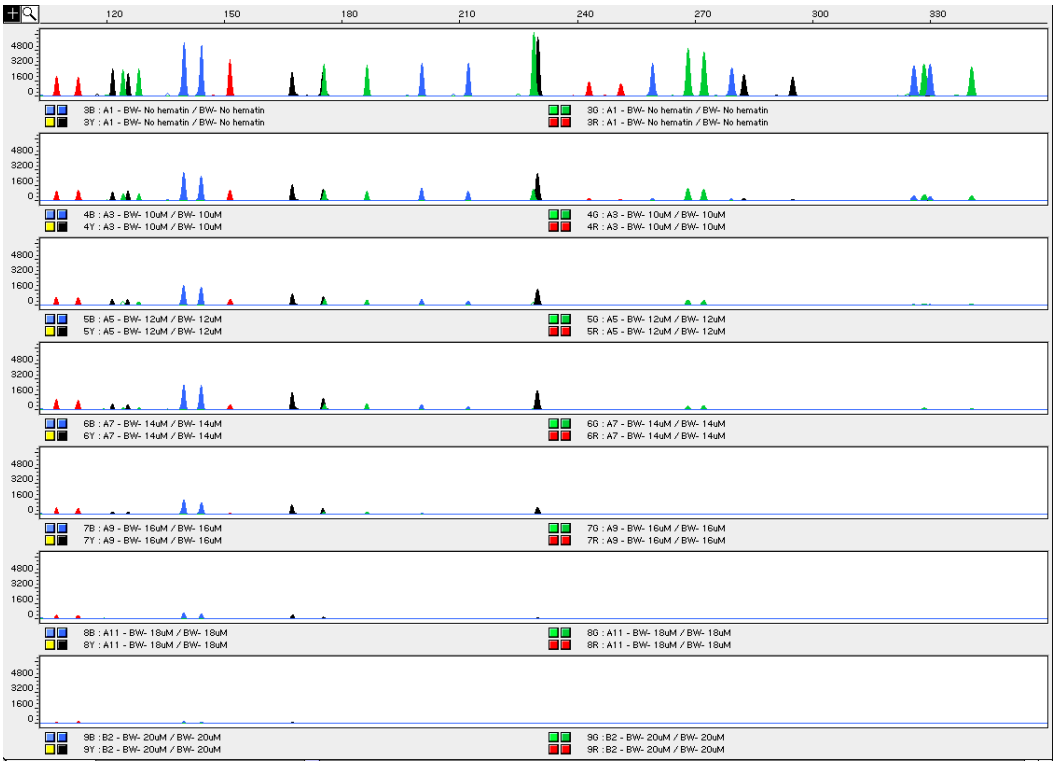

120
150
180
210
240
270
300
330
4800
3200
1600
0
3B : A1 - BW- No hematin / BW- No hematin
3Y : A1 - BW- No hematin / BW- No hematin
3G : A1 - BW- No hematin / BW- No hematin
3R : A1 - BW- No hematin / BW- No hematin
4B : A3 - BW- 10uM / BW- 10uM
4Y : A3 - BW- 10uM / BW- 10uM
4G : A3 - BW- 10uM / BW- 10uM
4R : A3 - BW- 10uM / BW- 10uM
5B : A5 - BW- 12uM / BW- 12uM
5Y : A5 - BW- 12uM / BW- 12uM
5G : A5 - BW- 12uM / BW- 12uM
5R : A5 - BW- 12uM / BW- 12uM
6B : A7 - BW- 14uM / BW- 14uM
6Y : A7 - BW- 14uM / BW- 14uM
6G : A7 - BW- 14uM / BW- 14uM
6R : A7 - BW- 14uM / BW- 14uM
7B : A9 - BW- 16uM / BW- 16uM
7Y : A9 - BW- 16uM / BW- 16uM
7G : A9 - BW- 16uM / BW- 16uM
7R : A9 - BW- 16uM / BW- 16uM
8B : A11 - BW- 18uM / BW- 18uM
8Y : A11 - BW- 18uM / BW- 18uM
8G : A11 - BW- 18uM / BW- 18uM
8R : A11 - BW- 18uM / BW- 18uM
9B : B2 - BW- 20uM / BW- 20uM
9Y : B2 - BW- 20uM / BW- 20uM
9G : B2 - BW- 20uM / BW- 20uM
9R : B2 - BW- 20uM / BW- 20uM

Figure 4-13 DNA amplified with the AmpFℓSTR Identifiler kit in the presence of varying concentrations of hematin: 0, 10 µM, 12 µM, 14 µM, 16 µM, 18 µM, and 20 µM, analyzed on the ABI PRISM 310 Genetic Analyzer

Degraded DNA

As the average size of degraded DNA approaches the size of the target sequence, the amount of PCR product generated is reduced. This is due to the reduced number of intact templates in the size range necessary for amplification.

Degraded DNA was prepared to examine the potential for differential amplification of loci. High molecular weight DNA was incubated with the enzyme DNase I for varying amounts of time. The DNA was examined

by agarose gel analysis to determine the average size of the DNA fragments at each time point.

Four nanograms of degraded DNA (or 1 ng undegraded DNA) was amplified using the AmpFℓSTR Identifiler kit (all 16 primer pairs together). As the DNA became increasingly degraded the loci became undetectable according to size. Preferential amplification was not observed. The loci failed to robustly amplify in the order of decreasing size as the extent of degradation progressed: CSF1PO and D2S1338 were the first loci to exhibit decreased amplification, followed by D16S539 and D18S51 and so forth. A similar result at each time point was obtained whether the DNA samples were amplified for each locus alone or co-amplified with the AmpFℓSTR Identifiler kit (Figure 4-14).

Figure 4-14 Multiplex amplifications of a DNA sample in the absence of DNase I and the sample incubated for 30 sec, 1 min, 4 min, and 8 min with DNase I, analyzed using the ABI PRISM 310 Genetic Analyzer

Multiplex Amplifications

DNA samples were amplified in 16 separate reactions containing primers for only one AmpFℓSTR Identifiler kit locus (singleplex) and a

reaction containing all primers for the AmpFℓSTR Identifiler kit loci (multiplex). DNA used as PCR template consisted of a sample that had been degraded for 1 min with DNase I.

Amplified samples were analyzed using the ABI PRISM 310 Genetic Analyzer. Similar results were obtained (genotype and peak height) whether the DNA samples were amplified for each locus alone or co-amplified in the AmpFℓSTR Identifiler kit reaction, see Figure 4-15 on page 4-38.

When degraded DNA is suspected to have compromised amplification of one or more loci, the molecular weight of the DNA can be assessed by agarose gel analysis. If the DNA is degraded to an average of 400 base pairs in size or less, adding more DNA template to the AmpFℓSTR Identifiler kit amplification reaction may help produce a typeable signal for the loci. Adding more DNA to the amplification may provide more of the necessary size template for amplification.

125
150
175
200
225
250
275
300
325
350
1080
540
0
5B : A4 1 minute /
5Y : A4 1 minute /
5G : A4 1 minute /
5R : A4 1 minute /
16R : B6 AMEL / AMEL
12Y : B2 D19 / D19
7G : A5 D3 / D3
1B : A1 D8 / D8
17R : B7 D5 / D5
13Y : B3 vWA / vWA
8G : A6 THO1 / THO1
3B : A2 D21 / D21
18R : B8 FGA / FGA
9G : A7 D13 / D13
14Y : B4 TPOX / TPOX
4B : A3 D7 / D7
10G : A8 D16 / D16
15Y : B5 D18 / D18
6B : A4 CSF / CSF
11G : B1 D2 / D2

Figure 4-15 Multiplex and singleplex amplifications of a DNA sample incubated for 1 min with DNase I, analyzed on the ABI PRISM 310 Genetic Analyzer

Mixture Studies

8.1.2.2 Mixture Studies

"*Species specificity, sensitivity, stability and mixture studies are conducted.*" (DAB, 1998).

Evidence samples may contain DNA from more than one individual. The possibility of multiple contributors should be considered when interpreting the results. We recommend that individual laboratories assign a minimum peak height threshold based on validation experiments performed in each laboratory to avoid typing when stochastic effects are likely to interfere with accurate interpretation of mixtures.

Mixed Specimen Studies

Evidence samples that contain body fluids and/or tissues originating from more than one individual are an integral component of forensic casework. Therefore it is essential to ensure that the DNA typing system is able to detect DNA mixtures. In the case of STRs, stutter peaks may be informative in the interpretation of mixed samples. Furthermore, alleles amplified with the AmpFℓSTR Identifiler kit have similar peak height values for a heterozygous genotype within a locus. This balance can be used as an aid in detecting and interpreting mixtures.

Detection of Mixed Samples

Each of the following can aid in determining whether a sample is a mixture:

- The presence of greater than two alleles at a locus
- The presence of a peak at a stutter position that is significantly greater in percentage than what is typically observed in a single-source sample
- Significantly imbalanced alleles for a heterozygous genotype

  The peak height ratio is defined as the height of the lower peak (in RFU) divided by the height of the higher peak (in RFU), expressed as a percentage. Mean, median, minimum and maximum peak height ratios observed for alleles in the AmpFℓSTR Identifiler kit loci in unmixed population database samples are as follows:

Table 4-2 Peak Height Ratios

Allele	Number of Observations (n)	Mean*	Median*	Minimum*	Maximum*
CSF1PO	84	86	88	63.6	99.8
D2S1338	93	84	86	42.8	99.7
D3S1358	91	88	90	64.3	99.7
D5S818	82	89	91	64.9	99.7
D7S820	96	89	90	66.2	99.5
D8S1179	89	90	93	57.5	99.8
D13S317	96	87	87	63.3	100.0
D16S539	92	88	91	61.5	99.9
D18S51	99	82	83	56.3	99.9
D19S433	98	88	92	48.8	100.0
D21S11	92	88	89	66.4	99.6
FGA	94	85	87	60.9	99.5
TH01	99	86	88	48.8	99.9
TPOX	87	87	92	55.9	99.8
vWA	101	86	88	62.8	99.1

*Peak height ratios were determined for those heterozygous samples with peak heights > 200 RFU.

For all 15 loci, the mean peak height ratios indicate that the two alleles of a heterozygous individual are generally very well balanced.

If an unusually low peak height ratio is observed for one locus, and there are no other indications that the sample is a mixture, the sample may be reamplified and reanalyzed to determine if the imbalance is reproducible. Possible causes of imbalance at a locus are degraded DNA, presence of inhibitors, extremely low amounts of input DNA, or the presence of an allele containing a rare sequence that does not amplify as efficiently as the other allele.

Resolution of Genotypes in Mixed Samples

A sample containing DNA from two sources can be comprised (at a single locus) of any of the seven genotype combinations listed below.

- Heterozygote + heterozygote, no overlapping alleles (four peaks)

- Heterozygote + heterozygote, one overlapping allele (three peaks)
- Heterozygote + heterozygote, two overlapping alleles (two peaks)
- Heterozygote + homozygote, no overlapping alleles (three peaks)
- Heterozygote + homozygote, overlapping allele (two peaks)
- Homozygote + homozygote, no overlapping alleles (two peaks)
- Homozygote + homozygote, overlapping allele (one peak)

Specific genotype combinations and input DNA ratios of the samples contained in a mixture determine whether it is possible to resolve the genotypes of the major and minor component(s) at a single locus.

The ability to obtain and compare quantitative values for the different allele peak heights on Applied Biosystems instruments provides additional valuable data to aid in resolving mixed genotypes. This quantitative value is much less subjective than comparing relative intensities of bands on a stained gel.

Ultimately, the likelihood that any sample is a mixture must be determined by the analyst in the context of each particular case, including the information provided from known reference sample(s).

Limit of Detection of the Minor Component

Mixtures of two DNA samples were examined at various ratios (1:1 to 1:10). The total amount of genomic input DNA mixed at each ratio was 1 ng.

The samples were amplified in a GeneAmp® PCR System 9700 and were electrophoresed and detected using an ABI PRISM 310 Genetic Analyzer.

The results of the mixed DNA samples are shown in Figure 4-16, where sample A and sample B were mixed according to the ratios provided.

The profiles of the samples in Figure 4-16 are the following:

	Profile	
Allele	**Sample A**	**Sample B**
Amelogenin	X	X, Y
CSF1PO	10, 12	11,12
D2S1338	17, 25	20, 23
D3S1358	15, 18	15,16
D5S818	11, 13	11
D7S820	9, 10	7,12
D8S1179	13	12,13

	Profile	
Allele	**Sample A**	**Sample B**
D13S317	11	11
D16S539	11, 12	9, 10
D18S51	17, 19	12, 15
D19S433	13	14,15
D21S11	30, 30.2	28, 31
FGA	23.2, 24	24, 26
TH01	7, 9	7, 9.3
TPOX	8, 9	8
vWA	17, 19	14,16

For these 1-ng total DNA mixture studies, the limit of detection is when the minor component is present at approximately one-tenth of the concentration of the major component and a threshold of 50 RFU. The limit of detection for the minor component is influenced by the combination of genotypes in the mixture.

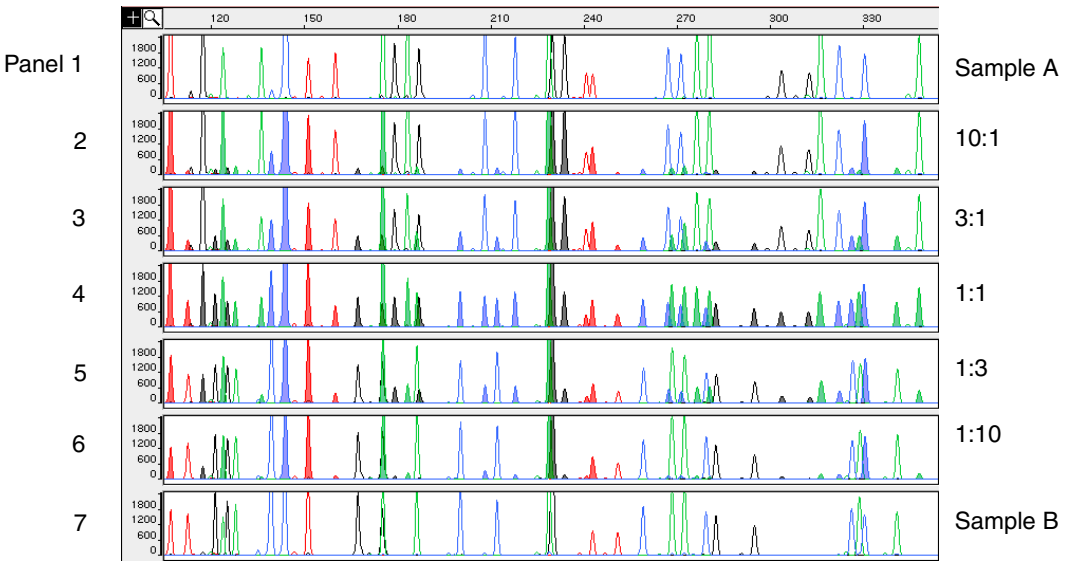


Figure 4-16 Results of the two DNA samples mixed together at defined ratios and amplified with the AmpFℓSTR Identifiler PCR Amplification Kit. Sample A and Sample B are a female and male sample, respectively. The ratios of Sample A to Sample B (A:B ratios) shown are 10:1, 3:1, 1:1, 1:3, and 1:10, respectively. The alleles attributable to the minor component, even when the major component shares an allele, are highlighted in panels 2, 3, 5, and 6. All alleles are highlighted in panel 4.

Data Interpretation

Minimum Sample Requirement

The AmpFℓSTR Identifiler PCR Amplification Kit has been optimized to amplify and type approximately 0.5–1.25 ng of sample DNA reliably.

The PCR cycle number and amplification conditions have been specified to produce low peak heights for a sample containing 20 pg human genomic DNA. Thus, the overall sensitivity of the assay has been adjusted to avoid or minimize stochastic effects. Applied Biosystems has successfully typed samples containing less than 0.5 ng DNA.

Note Individual laboratories may find it useful to determine an appropriate minimum peak height threshold based on their own results/instruments using low amounts of input DNA.

Population Data

8.1.2.3 Population Data

"*Population distribution data are documented and available.*" (DAB, 1998).

8.1.2.3.1 Population Distribution Data

"*The population distribution data would include the allele and genotype distributions for the locus or loci obtained from relevant populations. Where appropriate, databases should be tested for independence expectations."* (DAB, 1998).

Overview

To interpret the significance of a match between genetically typed samples, it is necessary to know the population distribution of alleles at each locus in question. If the genotype of the relevant evidence sample is different from the genotype of the suspect's reference sample, then the suspect is "excluded" as the donor of the biological evidence tested. An exclusion is independent of the frequency of the two genotypes in the population.

If the suspect and evidence samples have the same genotype, then the suspect is "included" as a possible source of the evidence sample. The probability that another, unrelated, individual would also match the evidence sample is estimated by the frequency of that genotype in the relevant population(s).

Population Samples Used in These Studies

The AmpFℓSTR Identifiler PCR Amplification Kit, prior to the addition of the D8S1179 degenerate primer, was used to generate the population data provided in this section. Samples were collected from individuals throughout the United States with no geographical preference.

African-American

357 samples were provided by the Kentucky State Police and the Federal Bureau of Investigation.

U.S. Caucasian

349 samples were provided by the Kentucky State Police and the Federal Bureau of Investigation.

U.S. Hispanic

290 samples were provided by the Minnesota Bureau of Criminal Apprehension/Memorial Blood Center of Minneapolis and the Federal Bureau of Investigation.

Native American

191 samples were provided by the Minnesota Bureau of Criminal Apprehension/Memorial Blood Center of Minneapolis.

AmpFℓSTR Identifiler Kit Allele Frequencies

Table 4-3 shows the AmpFℓSTR Identifiler kit allele frequencies in four populations, listed as percentages.

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
CSF1PO				
6	*	*	*	*
7	4.62	0.14*	0.34*	*
8	7.56	0.29*	0.17*	0.52*
9	3.78	1.72	0.86*	8.38
10	27.87	24.21	23.10	30.89
11	20.59	31.81	28.28	21.99
11.3	0.14*	*	*	*
12	29.13	32.81	39.66	32.72
13	5.32	7.31	6.38	4.71
14	0.98	1.43	0.86*	0.79*
15	*	0.29*	0.34*	*
D2S1338				
15	0.14*	*	*	*
16	5.32	4.73	2.41	2.62
17	10.78	17.34	21.21	9.95
18	5.60	6.30	4.14	7.07
19	14.15	13.75	22.76	29.58
20	6.02	14.61	13.79	9.69
21	14.01	2.58	2.59	2.36
22	13.17	4.01	7.41	15.18
23	10.78	11.46	11.38	11.78
24	9.80	11.75	8.45	7.85
25	8.12	10.60	5.17	3.14
26	1.96	2.72	0.69*	0.79*
27	0.14*	0.14*	*	*
28	*	*	*	*

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
D3S1358				
<11	0.42*	0.14*	*	*
11	*	*	*	0.26*
12	0.56*	*	0.17*	*
13	0.70*	0.29*	0.17*	*
14	12.04	15.76	7.41	6.81
15	30.53	25.36	39.14	40.84
15.2	0.14*	*	*	*
16	28.57	22.78	26.72	32.98
17	19.47	18.19	16.03	9.95
18	6.72	16.48	8.97	8.38
19	0.84	1.00	1.03	0.79*
20	*	*	0.34*	*
D5S818				
7	0.14*	*	6.72	15.71
8	5.46	*	0.69*	*
9	1.68	4.15	5.17	6.02
10	6.72	5.44	5.17	4.19
11	25.49	39.26	39.14	41.10
12	36.41	35.24	29.31	23.30
13	21.57	15.47	12.59	9.42
14	2.38	0.14*	0.69*	0.26*
15	*	0.29*	0.18*	*
16	*	*	0.17*	*
17	0.14*	*	0.17*	*

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
D7S820				
6	*	0.14*	0.17*	*
7	0.42*	1.29	1.72	0.52*
8	18.77	16.48	11.72	13.09
9	13.73	17.62	6.21	8.12
10	34.45	27.22	27.41	21.99
11	19.89	18.05	28.79	28.80
12	10.78	14.76	20.17	24.08
13	1.54	3.72	3.45	3.40
14	0.42*	0.72	0.34*	*
15	*	*	*	*
D8S1179				
8	0.42*	2.29	0.34*	0.52*
9	0.42*	1.15	0.34*	0.26*
10	2.38	9.74	8.45	4.71
11	3.92	6.02	5.86	3.40
12	13.31	14.04	12.07	11.52
13	23.25	32.52	32.93	37.43
14	30.11	21.35	26.21	30.63
15	20.17	9.89	10.86	9.42
16	4.62	2.72	2.41	1.57
17	1.12*	0.29*	0.52*	0.52*
18	0.28*	*	*	*
19	*	*	*	*
D13S317				
8	3.08	12.18	9.66	4.97
9	2.52	7.74	21.72	17.80
10	3.78	4.44	9.14	13.61
11	24.51	29.80	23.10	24.35
12	46.22	30.80	20.86	23.04
13	15.41	11.17	10.17	7.85
14	4.34	3.72	5.34	8.12
15	0.14*	0.14*	*	0.26*

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
D16S539				
5	*	*	*	*
8	3.22	1.72	1.72	0.79*
9	19.05	10.46	9.31	12.30
10	10.92	5.59	15.69	15.45
11	31.51	31.95	30.17	30.89
12	18.77	30.23	29.48	27.75
13	14.85	16.76	11.55	10.73
14	1.54	3.01	2.07	2.09
15	0.14*	0.29*	*	*
D18S51				
7	*	*	*	*
9	0.14*	*	*	*
10	0.28*	0.86	0.52*	0.79*
10.2	0.14*	*	*	*
11	0.28*	1.15	1.21*	*
12	7.00	13.90	10.34	14.92
13	4.34	12.18	14.48	9.16
13.2	0.42*	*	*	*
14	6.86	16.76	15.52	26.96
14.2	0.28*	*	*	*
15	19.47	13.61	16.55	12.04
16	16.53	13.61	11.72	10.73
17	18.21	12.32	14.14	14.66
18	11.90	7.74	6.72	2.62
19	6.02	4.44	4.14	3.93
20	4.90	1.72	2.24	1.83
21	2.10	1.00	1.03	1.31
22	0.70*	0.43*	0.52*	0.79*
23	0.42*	0.14*	0.52*	0.26*
24	*	0.14*	0.17*	*
25	*	*	0.17*	*
26	*	*	*	*
27	*	*	*	*

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
D19S433				
9	*	0.14*	0.17*	*
10	1.54	*	*	*
11	7.14	0.72	0.52*	0.52*
11.2	0.14*	*	0.17*	*
12	10.78	7.74	6.21	3.14
12.2	6.30	0.57*	1.90	*
13	29.83	28.94	16.03	17.80
13.2	5.74	1.72	8.62	15.45
14	21.01	34.10	31.72	24.87
14.2	4.20	0.86	5.00	3.66
15	4.76	15.76	13.45	13.35
15.2	3.36	2.72	8.79	10.73
16	2.38	4.15	4.31	3.93
16.2	2.38	1.72	2.93	1.83
17	*	0.29*	0.17*	0.79*
17.2	0.28*	0.29*	*	2.88
18.2	0.14*	0.29*	*	1.05*

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
D21S11				
24	*	*	*	*
24.2	0.14*	0.43*	0.17*	*
24.3	0.28*	*	*	*
25	*	*	*	*
25.2	*	0.14*	0.17*	*
26	0.14*	0.14*	0.17*	*
27	5.04	4.58	1.21	0.52*
28	22.97	16.76	9.14	6.28
28.2	*	*	*	*
29	19.33	20.49	21.21	16.75
29.2	0.14*	*	0.52*	0.26*
29.3	0.14*	*	*	*
30	17.23	25.21	29.31	34.29
30.2	1.40	3.30	2.93	1.83
31	7.98	7.16	6.72	5.76
31.2	7.98	9.46	8.62	18.85
32	1.12	1.43	1.55	0.79*
32.2	5.88	7.16	12.93	9.69
33	0.56*	*	*	0.52*
33.2	3.78	3.30	4.14	3.66
34	1.26	*	*	*
34.1	0.14*	*	*	*
34.2	0.14*	0.29*	0.86*	0.79*
35	2.94	*	0.34*	*
35.1	0.14*	*	*	*
35.2	*	0.14*	*	*
36	0.84	*	*	*
37	0.28*	*	*	*
38	0.14*	*	*	*

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
FGA				
16	*	0.14*	*	*
16.1	0.14*	*	*	*
17	*	0.29*	0.17*	*
17.2	0.14*	*	*	*
18	0.70*	2.72	0.52*	1.31
18.2	1.40	*	*	*
19	6.72	6.16	7.07	10.21
19.2	0.28*	*	*	*
20	7.00	13.90	7.41	12.30
20.2	*	0.14*	*	*
21	12.89	16.91	14.66	12.83
21.2	*	0.29*	0.17*	*
22	21.57	16.91	17.24	10.47
22.2	0.28*	1.29	0.34*	0.26*
22.3	0.14*	*	*	*
23	14.99	15.19	11.90	15.97
23.2	0.14*	*	0.86*	0.26*
24	17.51	13.75	15.34	15.71
24.2	*	0.14*	0.17*	*
25	7.98	8.60	14.14	14.14
26	3.50	2.72	6.90	4.45
26.2	*	*	*	0.52
27	1.82	0.72	2.41	0.79*
28	1.40	0.14*	0.69*	0.52*
29	0.56*	*	*	*
30	*	*	*	*
30.2	0.14*	*	*	*
31.2	*	*	*	*
32.2	*	*	*	*
33.2	*	*	*	*
34.2	0.14*	*	*	*
42.2	*	*	*	*
43.2	*	*	*	*

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
FGA, con't				
44.2	0.28*	*	*	*
45.2	*	*	*	0.26*
46.2	0.14*	*	*	*
47.2	*	*	*	*
48.2	0.14*	*	*	*
50.2	*	*	*	*
51.2	*	*	*	*
TH01				
4	*	*	*	*
5	0.28*	0.43*	0.17*	*
6	11.06	20.49	22.76	20.68
7	42.86	21.78	33.62	43.98
8	20.73	11.46	8.45	5.24
8.3	*	0.14*	*	*
9	12.32	16.19	14.14	6.28
9.3	11.62	29.08	20.34	23.56
10	0.98	0.43*	0.52*	0.26*
11	*	*	*	*
13.3	0.14*	*	*	*
TPOX				
6	6.72	0.14*	0.34*	*
7	2.24	*	0.34*	0.26*
8	36.13	53.30	49.66	37.96
9	21.15	11.60	7.24	4.19
10	9.24	4.30	4.66	3.40
11	21.43	25.93	27.24	39.27
12	3.08	4.73	10.52	14.92
13	*	*	*	*

Table 4-3 AmpFℓSTR Identifiler kit allele frequencies *(continued)*

Allele	African-American (n = 357)	U.S. Caucasian (n = 349)	U.S. Hispanic (n = 290)	Native American (n = 191)
vWA				
11	0.28*	*	0.17*	*
12	*	*	*	0.26*
13	1.26	0.43*	*	0.26*
14	7.14	8.31	6.90	4.45
15	20.03	11.32	10.00	7.07
16	26.75	23.35	34.31	32.98
17	20.59	24.50	21.55	33.51
18	14.71	22.49	18.45	15.45
19	6.72	8.31	7.07	4.71
20	1.96	1.15	1.38	1.05*
21	0.28*	*	0.17*	0.26*
22	0.28*	*	*	*
23	*	*	*	*
24	*	0.14*	*	*

*A minimum allele frequency (0.7% for the African-American database, 0.7% for the U.S. Caucasian database, 0.9% for the U.S. Hispanic database, and 1.3% for the Native American database) is suggested by the National Research Council in forensic calculations.

Analyzing the Four Databases

Analysis across the four databases of 2274 total chromosomes per locus revealed the following number of different alleles: 10 CSF1PO alleles, 13 D2S1338 alleles, at least 12 D3S1358 alleles, 11 D5S818 alleles, 9 D7S820 alleles, 11 D8S1179 alleles, 8 D13S317 alleles, 8 D16S539 alleles, 20 D18S51 alleles, 17 D19S433 alleles, 26 D21S11 alleles, 31 d FGA alleles, 9 TH01 alleles, 7 different TPOX alleles, and 13 vWA alleles.

In addition to the alleles that were observed and recorded in the Applied Biosystems databases, other known alleles have been published or reported to us by other laboratories (see STRBase, www.cstl.nist.gov/div831/strbase).

Low Frequency Alleles

Some alleles of the AmpFℓSTR Identifiler kit loci occur at a low frequency. For these alleles, a minimum frequency (five divided by 2n, where n equals the number of individuals in the database) was

assigned for the AmpFℓSTR Identifiler kit African-American, U.S. Caucasian, U.S. Hispanic and Native American databases, as suggested in the 1996 report of the Committee on DNA Forensic Science (National Research Council, 1996). These databases are summarized in Table 4-3. The minimum reportable genotype frequency at each locus is as follows: 1.19×10^{-4} for the African-American database; 1.19×10^{-4} for the U.S. Caucasian database; 1.70×10^{-4} for the U.S. Hispanic database; and 2.97×10^{-4} for the Native American database [$p^2 + p(1-p)\,\overline{\theta}$, where $\overline{\theta} = 0.01$]. Hence, the minimum combined multilocus genotype frequency at 15 loci is as follows: 1.36×10^{-59} for the African-American database; 1.36×10^{-59} for the U.S. Caucasian database; 2.86×10^{-57} for the U.S. Hispanic database; and 1.23×10^{-53} for the Native American database.

Mutation Rate

Estimating Germline Mutations

Estimation of spontaneous or induced germline mutation at genetic loci may be achieved through comparison of the genotypes of offspring to those of their parents. From such comparisons the number of observed mutations are counted directly.

In previous studies, genotypes of ten STR loci amplified by the AmpFℓSTR SGM Plus PCR Amplification Kit were determined for a total of 146 parent-offspring allelic transfers (meioses) at the Forensic Science Service, Birmingham, England. One length-based STR mutation was observed at the D18S11 locus; mutation was not detected at any of the other nine STR loci. The D18S11 mutation was represented by an increase of one 4-bp repeat unit, a 17 allele was inherited as an 18 (single-step mutation). The maternal/paternal source of this mutation could not be distinguished.

Additional Mutation Studies

Additional studies (Edwards *et al.*,1991; Edwards *et al.*,1992; Weber and Wong, 1993; Hammond *et al.*,1994; Brinkmann *et al.*,1995; Chakraborty *et al.*,1996; Chakraborty *et al.*,1997; Brinkmann *et al.*,1998; Momhinweg *et al.*,1998; Szibor *et al.*,1998) of direct mutation rate counts produced:

- Larger sample sizes for some of the AmpFℓSTR Identifiler kit loci.
- Methods for modifications of these mutation rates (to infer mutation rates indirectly for those loci where these rates are not large enough to be measured directly and/or to account for those events undetectable as Mendelian errors).

Probability of Identity

Table of Probability of Identity

Table 4-4 shows the Probability of Identity (P_I) values of the AmpFℓSTR Identifiler kit loci individually and combined.

Table 4-4 Probability of Identity values for the AmpFℓSTR Identifiler kit STR loci

Locus	African-American	U.S. Caucasian	U.S. Hispanic	Native American
CSF1PO	0.079	0.132	0.141	0.123
D2S1338	0.023	0.027	0.038	0.043
D3S1358	0.097	0.076	0.112	0.158
D5S818	0.104	0.147	0.115	0.110
D7S820	0.085	0.063	0.083	0.081
D8S1179	0.074	0.064	0.089	0.104
D13S317	0.132	0.079	0.056	0.056
D16S539	0.077	0.097	0.090	0.082
D18S51	0.033	0.031	0.031	0.046
D19S433	0.042	0.087	0.049	0.044
D21S11	0.037	0.044	0.047	0.074
FGA	0.034	0.035	0.032	0.031
TH01	0.109	0.079	0.097	0.134
TPOX	0.089	0.188	0.168	0.159
vWA	0.066	0.066	0.080	0.103
Combined	1.31×10^{-18}	5.01×10^{-18}	7.65×10^{-18}	3.62×10^{-17}

The P_I value is the probability that two individuals selected at random will have an identical AmpFℓSTR Identifiler kit genotype (Sensabaugh, 1982). The P_I values for the populations described in this section are then approximately $1/7.64 \times 10^{17}$ (African-American), $1/2.00 \times 10^{17}$ (U.S. Caucasian), $1/1.31 \times 10^{17}$ (U.S. Hispanic), and $1/2.76 \times 10^{16}$ (Native American).

Probability of Paternity Exclusion

Table of Probability of Paternity Exclusion

Table 4-5 shows the Probability of Paternity Exclusion (P_E) values of the AmpFℓSTR Identifiler kit STR loci individually and combined.

Table 4-5 Probability of paternity exclusion for the AmpFℓSTR Identifiler kit STR loci

Locus	African-American	U.S. Caucasian	U.S. Hispanic	Native American
CSF1PO	0.545	0.496	0.450	0.409
D2S1338	0.748	0.725	0.671	0.399
D3S1358	0.591	0.630	0.495	0.510
D5S818	0.506	0.440	0.525	0.601
D7S820	0.591	0.582	0.574	0.492
D8S1179	0.580	0.680	0.599	0.601
D13S317	0.383	0.487	0.638	0.370
D16S539	0.649	0.566	0.567	0.428
D18S51	0.760	0.731	0.767	0.329
D19S433	0.601	0.531	0.678	0.360
D21S11	0.737	0.708	0.586	0.399
FGA	0.760	0.766	0.739	0.309
TH01	0.492	0.566	0.618	0.646
TPOX	0.521	0.329	0.392	0.687
vWA	0.709	0.625	0.555	0.528
Combined	0.9999996	0.9999992	0.9999990	0.9999527

The P_E value is the probability, averaged over all possible mother-child pairs, that a random alleged father will be excluded from paternity after DNA typing of the AmpFℓSTR Identifiler kit STR loci (Chakraborty and Stivers, 1996).

Genotyping for the Macintosh OS

5

Overview

About This Chapter

This chapter describes the use of ABI PRISM® Genotyper® Software v2.5.2 in conjunction with the AmpFℓSTR® Identifiler™ Kit Template and the Macintosh® OS to automatically genotype samples.

In This Chapter

This chapter contains the following topics:

Topic	See Page
Overview	5-1
Using Genotyper Software for Automated Genotyping	5-2
Understanding the AmpFℓSTR Identifiler Kit Template File	5-10
Determining Genotypes	5-17

Using Genotyper Software for Automated Genotyping

About the Software

Genotyper® software is used to convert allele sizes obtained from ABI PRISM® GeneScan® Analysis Software into allele designations automatically and to build tables containing the genotype information. Genotypes are assigned by comparing the sizes obtained for the unknown sample alleles with the sizes obtained for the alleles in the allelic ladder.

A Genotyper software template file that contains macros specifically written for use with the AmpFℓSTR® Identifiler™ PCR Amplification Kit is provided with this manual and should be used with AmpFℓSTR Identifiler kit data. Install the template onto your computer following the instructions in the “READ_ME” file.

Note You must have Genotyper Software v2.5.2 or higher to run the AmpFℓSTR Identifiler Kit Template. It is recommended that this version of Genotyper software be run on a Power Macintosh computer with Macintosh OS 8.x or 9.1. Refer to the *Genotyper Software User’s Manual* (Electronic Document #904648) and *Genotyper Applications Tutorials* (Electronic Document #904649) for more detailed information about the Genotyper software. The Human Identification Tutorial and HID template file included with the Genotyper software v2.5.2 software package are for tutorial purposes only.

Before Running Genotyper Software

GeneScan Analysis Software sample data (particularly the allelic ladder) must meet a few specific requirements before the macros in the AmpFℓSTR Identifiler Kit Template can be used. These requirements are described in this section.

Sample Info

All samples must have a unique sample description in the Sample Info column of the GeneScan software sample sheet so that the macros in the AmpFℓSTR Identifiler Kit Template can build a table. Samples with an empty Sample Info field will not be incorporated into the table of genotypes. Also, lanes or injections that contain the AmpFℓSTR Identifiler Allelic Ladder must have the word “ladder” in the Sample Info. The first lane or injection of “ladder” that is found is the one that is used by the Kazam macro in the AmpFℓSTR Identifiler Kit Template to determine the sizes in the allele categories that will be used for genotyping.

It is possible to skip the first lane or injection of allelic ladder and use the second lane or injection of allelic ladder for genotyping instead. After importing the sample files, but before running the Kazam macro, remove the word “ladder” from the Sample Info in all four sample dye colors for the first lane or injection of allelic ladder in the Dye/lanes

window. Make sure that the word “ladder” is entered for Sample Info in the second lane or injection of allelic ladder. See step 3 on page 5-4 for a description of how to access the Sample Info field in the Dye/lanes window.

GeneScan Analysis Software Peak Recognition

All allele peaks in the allelic ladder for each locus must be “recognized” (labeled) in the GeneScan Analysis Software (*i.e.*, each allele peak must have an entry in the GeneScan table). Thus, all allele peaks in each allelic ladder must have a peak height value in relative fluorescence units (RFU) that is greater than the Peak Amplitude Threshold (PAT) that was specified in the GeneScan software Analysis Parameters. Also, all allele peaks in each allelic ladder must be resolved. For example, the FGA 26, 26.2 and 27 alleles must be resolved such that each peak has an entry in the GeneScan software table.

Sample allele peak heights must also be greater than the GeneScan Software PAT in order to be recognized (labeled) by Genotyper software. Note that the PAT value specified in the GeneScan software Analysis Parameters is not necessarily the same as the RFU value that may be used by the forensic analyst as the “interpretational threshold”. The “Low Signal” column of the appropriate Genotyper software table (see page 5-8) can be used to identify peaks that are greater than the GeneScan software PAT, but less than a specified minimum threshold (default 150 RFU in the table macro).

AmpFℓSTR Identifiler Kit Template

The AmpFℓSTR Identifiler Kit Template contains macros that perform the following steps automatically:

- Finds the lane or injection containing the allelic ladder
- Creates allele size categories that are centered on the sizes obtained for the allelic ladder alleles
- Assigns the appropriate allele label to sample alleles that size within the allele size categories
- Removes labels from stutter peaks by applying a filter
- Builds a table containing genotypes for all samples

Using the AmpFℓSTR Identifiler Kit Template File

Use the following procedure to assign genotypes to AmpFℓSTR Identifiler kit alleles automatically.

To use the AmpFℓSTR Identifiler Kit Template:

Step	Action
1	Double-click the **IDENTIFILER** icon to launch the Genotyper software application and open the template file simultaneously. **Note** The AmpFℓSTR Identifiler Kit Template is a Stationery pad, which means that a new document is created when the template file is opened. The original template file is not overwritten.
2	Set preferences to import raw data, Blue, Green, Yellow, Red and Orange.
3	To import the GeneScan sample files: a. Under the **File** menu, choose **Import GeneScan File**(s). b. Select the project file and click **Import**.
4	If each sample does not already have Sample Info completed in the sample sheet, this can be accomplished in Genotyper software as follows: a. Under the **Views** menu, choose **Show Dye/lanes** window. b. Select the first sample row by clicking on the row. c. Click the mouse cursor in the **Sample Info** box at the top of the window, and type the sample designation or description. d. Repeat steps b and c to enter a sample description for every dye/lane in the list. Enter the same sample description for all dye colors of a single sample.
5	From the **Macro** list at the bottom left of the **Main** window, select **Check GS500**.
6	Under the **Macro** menu, choose **Run Macro**. In the plot window that appears, scroll through each sample to verify that each GeneScan-500 peak (from 75–450 bp) was assigned the correct size in the GeneScan Analysis Software.
7	From the **Macro** list at the bottom left of the **Main** window, select Kazam.
8	Under the **Macro** menu, choose **Run Macro**. This macro may take a few minutes to run. When it is finished, a plot window opens with the blue allelic ladder (D8S1179, D21S11, D7S820, and CSF1PO) and sample allele peaks labeled.
9	Examine data and edit peaks.
10	Print the electropherograms in the plot window by choosing **Print** from the **File** menu.

To use the AmpFℓSTR Identifiler Kit Template: *(continued)*

Step	Action
11	a. In the **Main Window**, click the green G button at the top left. b. From the **Views** menu, select **Show Plot Window**. c. Repeat steps 8 and 9.
12	a. In the **Main Window**, click the yellow **Y** button at the top left. b. From the **Views** menu, select **Show Plot Window**. c. Repeat steps 8 and 9.
13	a. In the **Main Window**, click the red R button at the top left. b. From the **VIews** menu, select **Show Plot Window**. c. Repeat steps 8 and 9.

Examining Data

Check that the peaks in the allelic ladder are labeled correctly. Scroll through the samples below the allelic ladder to examine the peak labels in each electropherogram.

Peak Labeling

- Allele categories (which appear as dark gray bars in the Plot window) are defined to be ±0.5 bp wide. Peaks that size within ±0.5 bp of an allele category will have a label indicating the allele designation.

Note The categories for TH01 alleles 9.3 and 10 are ± 0.4 bp wide.

- Peaks that do not size within an allele category will have a label indicating “OL Allele?” (off-ladder allele).
- The Kazam macro includes a step that removes labels from stutter peaks by applying a percentage filter. Labels are removed from peaks that are followed by a (specified percent difference) higher, labeled peak within 3.25 to 4.75 bp. The specified filter percentages for these loci are 1861% for TH01, 1983% for TPOX, 987% for CSF1PO, 1371% for D5S818, 1150% for D13S317, 835% for D3S1358, 694% for vWA, 580% for FGA, 1120% for D8S1179 and D7S820, 862% for D16S539, 964% for D21S11, 801% for D2S1338, 652% for D19S433 and 488% for D18S51. These filter percentages correspond to the upper-limit stutter percent values observed for each locus (see Figures 4-4 through 4-7).
- A sample allele peak must have been “recognized” by GeneScan software before it can be recognized by Genotyper software. Thus, sample allele peaks that are below the PAT that was specified in the GeneScan software Analysis Parameters cannot be labeled by Genotyper software.

  Also, because no information is imported for peaks that are not recognized by GeneScan software, such peaks will not align exactly by size relative to the x-axis size scale in the Genotyper software plot window.

Peak Editing

- Clicking on a labeled peak removes the label. Clicking again on the same peak defaults to the placement of bp size of that peak. A dialog box with a field to enter the requested text may be accessed by Edit menu, set click options. Type the allele designation and/or desired text, then click **OK**.

Plot Window Viewing Options

To zoom in and out on regions of the plot window:

Step	Action
1	Click and drag in a region of an electropherogram in the Plot window to draw a box around the desired size range (the vertical size of the box is not important).
2	Type ⌘ **R** (hold down the command key and type the letter R) to zoom in.
3	Type ⌘ **H** to zoom out completely.

To view electropherograms from more than one dye color in the Plot window:

Step	Action
1	Under the **Views** menu, choose **Show Dye/Lanes Window**.
2	Click on the desired Dye/lane rows. **Note** Hold down the Shift key on the keyboard to select multiple adjacent Dye/lane rows. Hold down the Command (⌘) key to select Dye/lane rows that are not adjacent.
3	Under the **Views** menu, choose **Show Plot Window**.

Making Tables

Three macros for making tables are included in the AmpFℓSTR Identifiler Kit Template. They are:

a. Make Allele Table

b. Make CODIS Table

c. 310: Make Table

Two of these tables, Make Allele Table and Make CODIS Table, are very simple and contain only Sample Info and genotype data. The other table, 310: Make Table contains additional information.

The contents and unique features of each table are described below. All four of the tables have two features in common:

- A locus that has no labeled peaks will have zeros in the cells of the table for that locus.
- Loci that have homozygous alleles will have the allele designation indicated twice in the table.

Make Allele Table

This table has Sample Info in the first column, and allele designations for each locus in columns 2–23. The first two labeled peaks within each locus appear in the table.

Make CODIS Table

This table has Sample Info in the first column, locus name in the second column, and allele designations in columns 3–4. This alternative table is to facilitate laboratories that will be importing data into the Combined DNA Index System (CODIS).

310: Make Table

This table can be used if the data was generated on the ABI PRISM 310 Genetic Analyzer. This table has Sample Info in the first column, Sample Comment in the second column, locus name in the third column, and allele designations in columns 4–7. Four columns are provided for allele designations to accommodate mixed samples. The first four labeled peaks within each locus appear in the table. The remaining five table columns are as follows:

- Overflow: If more than two peaks are labeled at one locus, the text "> two labels" will appear in this column.
- Low Signal: If the height of any peak at a locus is greater than the PAT specified in the GeneScan Analysis Parameters but less than 150 RFU, the text "< 150 RFU" will appear in this column.
- Saturation: If the raw data signal for any peak at a locus is greater than 8191 RFU, the text "310: off-scale" will appear in this column.
- Edited Label: The text "Edited" will appear in this column for any loci where the peak labels were edited manually. For example, clicking on an unlabeled peak in the Plot window to add a label constitutes an edit.
- Edited Row: The text "Edited" will appear in this column for any rows in the table that contain table cells that have been edited after initial creation of the table.

IMPORTANT Before making a table, all electropherograms should be examined and their peaks edited as described in the previous section.

To create and use tables:

Step	Action
1	From the **Macro** list at the bottom of the Genotyper software Main Window, click on one of the three table macros.
2	From the **Macro** menu, choose **Run Macro**.

To create and use tables: *(continued)*

Step	Action
3	Under the **Views** menu, select **Show Table Window** to view the table in full screen mode.
4	**For all tables except the Make Allele Table:**
	Clicking in a cell of the table causes the corresponding sample electropherogram to appear in the plot window, as follows: a. Click on any cell in the table. This causes this locus region of the corresponding electropherogram to appear in the Plot window for that sample. b. Zoom out (⌘ **H**) to view all loci for a particular dye color for the corresponding sample.
5	To edit the cells of the table: a. Click in a cell of the table that contains an allele designation. b. From the **Edit** menu, select **Edit Cell** c. Type the desired information in the box and click **OK**.
6	Print the table by selecting **Print** from the **File** menu.
7	**Optional**: From the **Table** menu, select **Export to File** to save the table as a Microsoft Excel-readable document.
8	From the **File** menu, select **Save** to save the template file with data.

Understanding the AmpFℓSTR Identifiler Kit Template

Troubleshooting Automated Genotyping

To Troubleshoot Automated Genotyping:

Observation	Probable Cause	Recommended Action
Warning message: "Could not complete 'Run Macro' command because no dye/lanes are selected".	The word "ladder" is not in Sample Info for the lane or injection of allelic ladder.	Type the word **Ladder** in Sample Info. The word Ladder must be entered for each dye color (Blue, Green, Yellow, and Red) in Sample Info for the AmpFℓSTR Identifiler Allelic Ladder sample.
Warning message: "Could not complete 'Run Macro' command because the labeled peak could not be found".	One or more peaks in the allelic ladder are below the Peak Amplitude Threshold that was specified in the GeneScan software Analysis Parameters.	Use another allelic ladder in the project, or a. In the GeneScan Analysis Software, lower the Peak Amplitude Threshold values for Blue, Green, Yellow and Red dye colors in the Analysis Parameters. b. Reanalyze the sample file(s) containing the allelic ladder. c. Import all sample files into a new Genotyper software project, and run the Kazam macro again.

About This Kit Template

This section describes the organization and functionality of the AmpFℓSTR Identifiler Kit Template. Read this section for a greater understanding of the macros and steps that are used in the AmpFℓSTR Identifiler Kit Template.

Categories

In the Genotyper software, each allele is defined by a category. Each category contains information about the allele size, size range, and dye color. To view the list of categories in the AmpFℓSTR Identifiler Template, choose Show Categories window, under the View menu. The categories for each locus are listed together under the locus name. The locus is called a group.

In the Categories window, each locus actually has two sets of categories. For example, the D3S1358 locus has one list of categories under the group "D3S1358" and another list of categories under the

group “D3S1358.os.” The categories in the D3S1358 group are allele categories and are used for allele assignment.

Offset Categories

As noted above, the offset values are determined automatically by the Calculate [locus] Offsets macros. These macros use the offset categories (categories with an “.os” suffix) to find the allele peaks in the allelic ladder and to determine the correct offset values for each allele category.

Finding and recognizing the Leftmost (first) Allele Peak in Each Allelic Ladder

- Identification of the leftmost peak is accomplished through the specifications of the first “.os” category listed within each group of offset categories. This first “.os” category (12.os in the case of D3S1358) is specified to find all peaks in a range of ±7 bp around the reference size for the indicated allele.
- Each Calculate [locus] Offsets macro applies a percentage filter to all peaks in the ±7-bp range in the allelic ladder. This avoids the first stutter peak in each allelic ladder and thus identifies the first allele peak as the leftmost peak.

Calculating the Offset Values

Categories with the “.os” suffix are called offset categories and are described below.

The base pair size indicated in each category is a “reference size.” One main function of the macros in the AmpFℓSTR Identifiler Template is to offset the reference sizes relative to the sizes obtained for the alleles in the allelic ladder. These offset steps are performed by the Calculate [locus] Offsets macros, located in the Macro list of the Genotyper software Main window. After the macros are run, the calculated offset values are indicated in parentheses near the end of each category line in the Categories window.

An example of how to interpret the offset values is given here for D3S1358 allele 14. The reference size for this allele is 122 bp. On a particular ABI PRISM 310 injection, the size obtained for D3S1358 allele 14 was 119.06 bp. The offset value is calculated as 119.06 – 122 = –2.94. In this example, the actual category size used for allele assignment is 119.06 (equals 122–2.94), which is the size of the D3S1358 allele 14 in this particular injection of the allelic ladder. In other words, the category sizes used for genotyping are equivalent to the allele sizes obtained in the lane or injection of allelic ladder.

Applying the Appropriate Offset Value to Each Allele in Succession

Once the leftmost allele peak in each allelic ladder is identified, the offset value determined for this allele is applied to the relevant allele(s) in the allele categories.

For example, assume that the offset value determined by the 12.os category in the D3S1358.os group is –3.01 for a particular lane or injection of allelic ladder. This offset value is then applied to the allele 12 category in the D3S1358 group, thus setting the correct offset value for allele 12.

In order for the software to find the next allele peak in the D3S1358 allelic ladder (allele 13), the offset value for the 12.os allele is also applied to the 13.os category. The result of this operation is that the 13.os category size will be 4 bp longer than the 12.os category. In other words, allele 13 is expected to be found at a size that is 4 bp longer than allele 12.

To maximize the ease of peak recognition, the size width for most offset categories is ±1 bp, as compared to the allele categories, which have a width of ±0.5 bp. Once allele 13 is recognized in the D3S1358 allelic ladder, the correct offset value is calculated and assigned to the appropriate categories.

This process of peak recognition, offset calculation, and offset assignment is carried out for each of the alleles in each of the allelic ladders.

Off-Ladder Alleles and Virtual Alleles

In the previous example, the 12.os offset value (-3.01) is also applied to two other categories in the D3S1358 group: “OL Allele?” and allele 11.

The OL Allele? category is specified to span the range of known D3S1358 alleles and is intended to catch off-ladder alleles that do not size within one of the allele categories.

Allele 11 in this case is a “virtual” allele category, meaning that this allele is not present in the allelic ladder. The virtual category exists to assign an allele designation to allele 11, which is a known allele not included in the allelic ladder.

Because allele 11 is specified to have the same offset value as allele 12, the allele category sizes for these two alleles will differ by exactly 4 bp, which is the same as the difference in their reference sizes. Specifying a size for allele 11 that is 4 bp shorter than allele 12 is generally expected to be a reasonable estimate since alleles 11 and 12 differ by a single repeat unit (4 bp).

The D3S1358 group also contains virtual allele categories for other alleles, such as 15.2 and 20. The offset value for allele 15.2 is specified to be the same as for allele 15. In this case, since reference sizes for these two alleles differ by 2 bp, the category size used for allele 15.2 will be 2 bp longer than for allele 15. Likewise, the offset for allele 20 is specified to be the same as for allele 19, so the allele category size for allele 20 will be 4 bp longer than for allele 19.

Many of the loci in the Categories window contain virtual allele categories. For example, the FGA locus contains a virtual category for many 2-bp length variants.

Kazam Macro

The Kazam macro is the top level macro that contains all of the instructions and steps necessary for determination of genotypes relative to the allelic ladder. Kazam references the Calculate [locus] Offsets macros for each locus, and contains further instructions to label peaks at each locus and to filter (remove labels from) the stutter peaks. The various steps in Kazam can be viewed in the Genotyper software by clicking on the Kazam line in the Macro list, and then choosing Show Step window, under the View menu.

Filtering Stutter Peaks

To illustrate the steps involved in filtering the stutter peaks, consider again the example of the D3S1358 locus:

To filter stutter peaks:

Step	Action
1	In the Step Window for the Kazam macro, scroll down to the line that reads “Select category: D3S1358”.
2	Five rows below, click on the line that reads “Remove labels from peaks followed by a 835% higher, labeled peak within 3.25 to 4.75 bp”.

To filter stutter peaks:

Step	Action
3	From the Macro menu, choose Edit Step. In the Filter Labels window that appears, there are four options (check boxes) for filtering. In this example, the filtering option for D3S1358 is denoted in the last check box. This filtering option includes another check box that reads “(higher by at least 835%).” For each labeled peak (*e.g.* peak A) in the locus size range, this filtering option examines the very next (*i.e.* greater in bp size) labeled peak (peak B). The label will be removed from peak A if peak B meets both of the specified criteria: a. peak B is higher by at least 835% b. peak B is within 3.25 to 4.75 bp The percentage value in this filtering option is calculated as follows: [(peak B – peak A) / peak A] x 100 = percentage value For example, if peak A = 175 RFU and peak B = 2500 RFU, then the percentage value is calculated as follows: [(2500 – 175) / 175] x 100 = 1329% In this example, the label will be removed from peak A, provided that the filter option specifies a threshold of 835% and that peak B is within 3.25 to 4.75 bp of peak A. Conventionally, percent stutter is calculated as described below: (peak A / peak B) x 100 = percent stutter The percentage value that is used in the Genotyper software filtering option (F) can be derived from the conventional percent stutter expression (S) as follows: F = (10,000 / S) – 100 For example, if the desired stutter percent threshold for D3S1358 is 11%, then the percentage value that should be used in the Genotyper software filtering option is: F = (10,000 / 11) – 100 = 835%
4	To use a filter value different than 835% for D3S1358, simply input another value.
5	Click **Replace**.

The peak filtering that is included in the Kazam macro is intended only as a tool and guideline. Final conclusions should be based on careful examination of the STR profiles.

Kazam (20% Filter)

The standard Kazam macro is written so that a different filter threshold can be used for each locus (the steps for each locus are written separately in the macro). The Kazam macro thus provides maximum flexibility and the opportunity to customize the filter that is used for each locus.

A different version of the Kazam macro called “Kazam (20% filter)” is also provided in the Macro list. This macro is simpler than the Kazam macro in that a 20% stutter filtering step is specified for all loci. To view the various steps in the Kazam (20% filter) macro, click on the Kazam (20% filter) line in the Macro list, and then choose “Show Step Window” from the “Views” menu. The first filter step for this macro (which applies to the sample alleles) reads, “Remove labels from peaks whose height is less than 20% of the highest peak in a category’s range.”

Note that this particular option does not include any condition regarding the bp size of the filtered peak relative to a higher peak. Indeed, this second filtering option will remove labels from all peaks that are less than a specified percentage of the highest peak observed anywhere in the locus range.

To edit the filter value:

Step	Action
1	Click on this step in the **Step** window.
2	Choose Edit Step from the Macro menu. **Note** This macro uses the second filter option in the Filter Labels window.
3	If desired, change the value from 20% to some other value
4	Click **Replace**.

The Kazam (20% filter) macro is provided as an option for laboratories that would like to use one general filter value for all loci. This macro can also be used when a high level of filtering specificity is not required, as in the typing of single source samples, *e.g.*, database samples.

Modifying the Template

The original AmpFℓSTR Identifiler Template File can be modified so that the changes made to the macros or settings are used as the default:

To modify the template:

Step	Action
1	Close all Genotyper windows, but do not quit the application.

To modify the template:

Step	Action
2	Find the **AmpFℓSTR Identifiler Kit Template** icon. Click on the icon to select it.
3	From the **File** menu, select **Get Info**.
4	a. Deselect the check box for Stationery pad at the bottom of the window. b. Close the **Get Info** window.
5	Open the **AmpFℓSTR Identifiler Kit Template** by double-clicking on its icon.
6	Make any desired changes.
7	Save the template file by choosing **Save** from the **File** menu.
8	Repeat steps 2 and 3.
9	a. Select the check box for Stationery pad at the bottom of the window. b. Close the Get Info window.

Determining Genotypes

AmpFℓSTR Identifiler Allelic Ladder

The AmpFℓSTR® Identifiler™ Allelic Ladder contains the most common alleles for each locus. Genotypes are assigned by comparing the sizes obtained for the unknown samples with the sizes obtained for the alleles in the allelic ladder.

Additional alleles have been included in the AmpFℓSTR Identifiler Allelic Ladder for the FGA, D18S51, D21S11,TH01, and vWA loci compared to those included for these same loci in other AmpFℓSTR kits.

The macro size ranges include the actual number of nucleotides contained in the smallest and largest allelic ladder alleles for each locus, as well as those alleles reported in STRBase (www.cstl.nist.gov/div831/strbase) as of September 2000. The size range also includes 3´ A nucleotide addition and any shifts due to non-nucleotide linkers (Grossman *et al*., 1994 and Baron *et al*., 1996). The non-nucleotide linkers enable reproducible positioning of the alleles to facilitate inter-locus spacing. The loci which incorporate these non-nucleotide linkers are as follows: CSF1PO, D2S1338, D13S317, D16S539 and TPOX. The AmpFℓSTR Identifiler PCR Amplification Kit is designed so that a majority of the PCR products contain the non-templated 3´ A nucleotide. The alleles have been named in accordance with the recommendations of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) (DNA Recommendations, 1994; Bar *et al*., 1997).

The number of complete four base pair repeat units observed is designated by an integer. Variant alleles that contain a partial repeat are designated by a decimal followed by the number of bases in the partial repeat. For example, an FGA 26.2 allele contains 26 complete repeat units and a partial repeat unit of two base pairs.

Additional variation has been seen at some loci where alleles exist that differ from integer allele lengths by one or three base pairs. For example, D21S11 allele 33.1 contains 33 complete repeat units and one nonconsensus base pair. Likewise, a D21S11 29.3 allele contains 29 complete repeat units and a partial 3-bp unit (Moller *et al.*, 1994; Gill *et al.*, 1997).

A Genotyper software electropherogram of the AmpFℓSTR Identifiler Allelic Ladder listing the designation for each allele is shown in Figure 5-1.

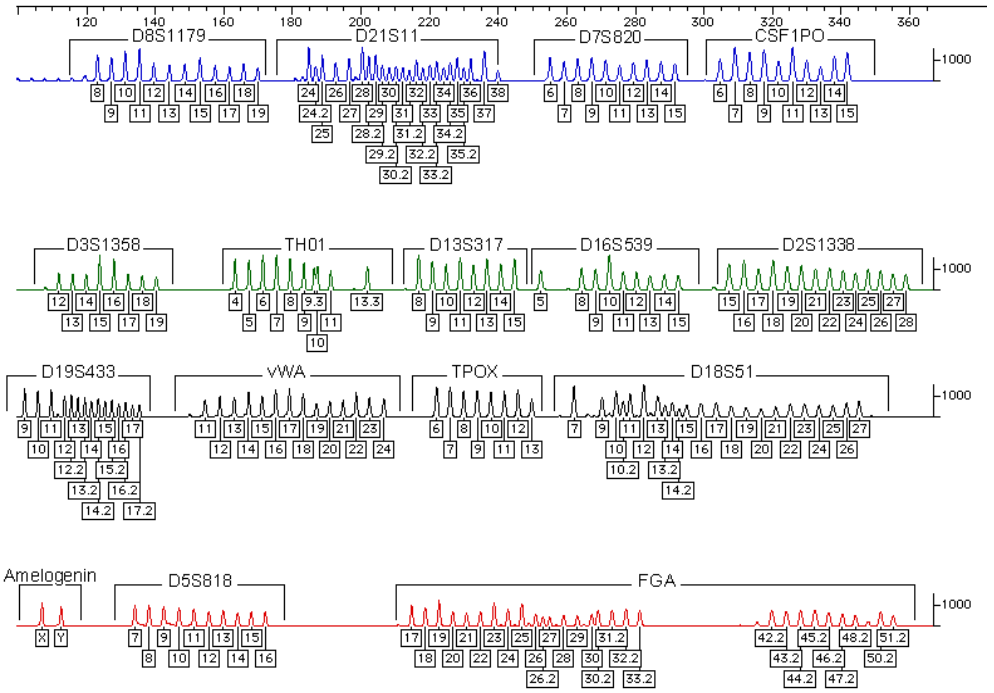


Figure 5-1 Genotyper® software plot of the AmpFℓSTR Identifiler Allelic Ladder, indicating the designation for each allele. These results were obtained on an ABI PRISM 310 Genetic Analyzer

Genotyping Using the AmpFℓSTR Identifiler Allelic Ladder

When interpreting AmpFℓSTR Identifiler kit results, genotypes are assigned to sample alleles by comparison of their sizes to those obtained for the known alleles in the AmpFℓSTR Identifiler Allelic Ladder. Genotypes, not sizes, are used for comparison of data between runs, instruments, and laboratories.

We strongly recommend that laboratories use an AmpFℓSTR Identifiler Allelic Ladder from each project to convert the allele sizes to genotypes. The main reasons for this approach are:

- The size values obtained for the same sample can differ between instrument platforms because of differences in the type and concentration of the gel/polymer matrices and in electrophoretic conditions.
- Sizes may differ between protocols for the same instrument platform because of differences in gel or polymer concentration, run temperature, gel or capillary thickness, and well-to-read length.
- Slight procedural and reagent variations between gels or between single and multiple capillaries result in greater size variation than that found between samples on the same gel or between samples injected in the same capillary in a single run.

Size Standard

The GeneScan-500 LIZ Size Standard should be used with the AmpFℓSTR Identifiler kit. Common alleles for all AmpFℓSTR Identifiler kit loci are less than 400 base pairs. The recommended sizing method, Local Southern, utilizes two internal lane size standard peaks larger than each allele and two smaller than each allele to be sized. Thus, when size standard peaks are defined in routine analyses, inclusion of the 400 base pair and 450 base pair peaks in the GeneScan-500 LIZ Size Standard is recommended.

The internal lane size standard is run with every sample (AmpFℓSTR Identifiler kit PCR products and AmpFℓSTR Identifiler Allelic Ladder) and is used to normalize lane-to-lane or injection-to-injection migration differences, thereby providing excellent sizing precision within a gel or within a set of capillary injections. Size windows based on the allelic ladder are used to assign allele designations to the samples. The procedure for running the allelic ladder and determining genotypes is described on the following page.

Analyzing AmpFℓSTR Identifiler Allelic Ladder

To size the AmpFℓSTR Identifiler Allelic Ladder alleles:

Step	Action
1	Analyze the lanes/injections containing allelic ladder using the same parameters used for samples.

Results

To compare the lanes or injections of AmpFℓSTR Identifiler Allelic Ladder:

Step	Action
1	Compare the base pair sizes of one lane or injection of allelic ladder to those obtained for the other lanes or injections of allelic ladder. All corresponding peaks (peaks at the same position in the allelic ladder) should be within ±0.5 bp of each other.
2	If one or more corresponding peaks are not within ±0.5 bp of each other: check the GeneScan-500 LIZ Size Standard peaks in all allelic ladder lanes or injections to confirm that all GeneScan-500 LIZ Size Standard peaks have been assigned the correct size and/or that all peaks are clearly resolved.

Genotyping Samples Manually

To manually genotype samples:

Step	Action
1	Select one lane or injection of allelic ladder to use for genotyping. **Note** Our studies have shown that it does not matter which lane or injection of allelic ladder is selected if the alleles in the allelic ladder samples are within ±0.5 bp of each other.
2	Compare the base pair size obtained for each sample allele peak to the sizes obtained for the allelic ladder peaks.
3	Assign genotypes to those sample allele peaks falling within ±0.5 bp of the corresponding allelic ladder peak. The allele designation for each allelic ladder peak is given in Figure 5-1 on page 5-18.

The AmpFℓSTR Identifiler Allelic Ladder contains the majority of alleles for the Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317,D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA loci. However, alleles not found in the

AmpFℓSTR Identifiler Allelic Ladder do exist. These “off-ladder” alleles may contain full and/or partial repeat units. An “off-ladder” allele should flag itself by not falling inside the ±0.5 bp window of any known allelic ladder allele.

Note If a sample allele peak is found to be ≥0.5 bp from the corresponding allelic ladder peak, then the sample must be rerun to verify the result.

Technical Support

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Contacting Technical Support

You can contact Applied Biosystems for technical support by telephone or fax, by e-mail, or through the Internet. You can order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents 24 hours a day. In addition, you can download documents in PDF format from the Applied Biosystems Web site (please see the section "To Obtain Documents on Demand" following the telephone information below).

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Contact technical support by e-mail for help in the following product areas:

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Sequence Detection Systems and PCR	pcrlab@appliedbiosystems.com
Protein Sequencing, Peptide and DNA Synthesis	corelab@appliedbiosystems.com
♦ Biochromatography ♦ PerSeptive DNA, PNA and Peptide Synthesis systems ♦ FMAT™ 8100 HTS System ♦ CytoFluor® 4000 Fluorescence Plate Reader ♦ Voyager™Mass Spectrometers ♦ Mariner™ Mass Spectrometers	tsupport@appliedbiosystems.com
Applied Biosystems/MDS Sciex	support@sciex.com
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Hours for Telephone Technical Support

In the United States and Canada, technical support is available at the following times:

Product	Hours
Chemiluminescence	8:30 a.m. to 5:30 p.m. Eastern Time
Framingham support	8:00 a.m. to 6:00 p.m. Eastern Time
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To contact Applied Biosystems Technical Support, use the telephone or fax numbers given below. (To open a service call for other support needs, or in case of an emergency, dial **1-800-831-6844** and press **1**.)

Product or Product Area	Telephone Dial...	Fax Dial...
ABI PRISM® 3700 DNA Analyzer	**1-800-831-6844,** then press **8**	**1-650-638-5981**
Fluorescent Fragment Analysis (includes GeneScan® applications)	**1-800-831-6844**, then press **2**, then **3**	**1-650-638-5981**
Integrated Thermal Cyclers (ABI PRISM®877 and Catalyst 800 instruments)	**1-800-831-6844**, then press **2**, then **4**	**1-650-638-5981**
ABI PRISM® 3100 Genetic Analyzer	**1-800-831-6844**, then press **2**, then **6**	**1-650-638-5981**
Peptide Synthesis (433 and 43X Systems)	**1-800-831-6844**, then press **3**, then **1**	**1-650-638-5981**
Protein Sequencing (Procise® Protein Sequencing Systems)	**1-800-831-6844**, then press **3**, then **2**	**1-650-638-5981**
PCR and Sequence Detection	**1-800-762-4001**, then press **1** for PCR, **2** for the 7700, 7900 or 5700, **6** for the 6700 or dial **1-800-831-6844**, then press **5**	**1-240-453-4613**

Product or Product Area	Telephone Dial...	Fax Dial...
♦ Voyager™ MALDI-TOF Biospectrometry ♦ Mariner™ ESI-TOF Mass Spectrometry Workstations	**1-800-899-5858**, then press **1**, then **3**	**1-508-383-7855**
Biochromatography (BioCAD® Workstations and POROS® Perfusion Chromatography Products)	**1-800-899-5858**, then press **1**, then **4**	**1-508-383-7855**
Expedite™ Nucleic acid Synthesis Systems	**1-800-899-5858**, then press **1**, then **5**	**1-508-383-7855**
Peptide Synthesis (Pioneer™ and 9050 Plus Peptide Synthesizers)	**1-800-899-5858**, then press **1**, then **5**	**1-508-383-7855**
PNA Custom and Synthesis	**1-800-899-5858,** then press **1**, then **5**	**1-508-383-7855**
♦ FMAT™ 8100 HTS System ♦ Cytofluor® 4000 Fluorescence Plate Reader	**1-800-899-5858**, then press **1**, then **6**	**1-508-383-7855**
Chemiluminescence (Tropix)	**1-800-542-2369** (U.S. only), or **1-781-271-0045**	**1-781-275-8581**
LC/MS Applied Biosystems/MDS Sciex	**1-800-952-4716**	**1-650-638-6223**

Outside North America

Region	Telephone Dial...	Fax Dial...
Africa and the Middle East		
Africa (English Speaking) and West Asia (Fairlands, South Africa)	**27 11 478 0411**	**27 11 478 0349**
Africa (French Speaking; Courtaboeuf Cedex, France)	**33 1 69 59 85 11**	**33 1 69 59 85 00**
South Africa (Johannesburg)	**27 11 478 0411**	**27 11 478 0349**

Region	Telephone Dial...	Fax Dial...
Middle Eastern Countries and North Africa (Monza, Italia)	**39 (0)39 8389 481**	**39 (0)39 8389 493**
Eastern Asia, China, Oceania		
Australia (Scoresby, Victoria)	**61 3 9730 8600**	**61 3 9730 8799**
China (Beijing)	**86 10 64106608** or **86 800 8100497**	**86 10 64106617**
Hong Kong	**852 2756 6928**	**852 2756 6968**
India (New Delhi)	**91 11 653 3743/3744**	**91 11 653 3138**
Korea (Seoul)	**82 2 593 6470/6471**	**82 2 593 6472**
Malaysia (Petaling Jaya)	**60 3 79588268**	**603 79549043**
Singapore	**65 896 2168**	**65 896 2147**
Taiwan (Taipei Hsien)	**886 2 2358 2838**	**886 2 2358 2839**
Thailand (Bangkok)	**66 2 719 6405**	**66 2 319 9788**
Europe		
Austria (Wien)	**43 (0)1 867 35 75 0**	**43 (0)1 867 35 75 11**
Belgium	**32 (0)2 532 4484**	**32 (0)2 582 1886**
Czech Republic and Slovakia (Praha)	**420 2 35365189**	**420 2 35364314**
Denmark (Naerum)	**45 45 58 60 00**	**45 45 58 60 01**
Finland (Espoo)	**358 (0)9 251 24 250**	**358 (0)9 251 24 243**
France (Paris)	**33 (0)1 69 59 85 85**	**33 (0)1 69 59 85 00**
Germany (Weiterstadt)	**49 (0) 6150 101 0**	**49 (0) 6150 101 101**
Hungary (Budapest)	**36 (0)1 270 8398**	**36 (0)1 270 8288**
Italy (Milano)	**39 (0)39 83891**	**39 (0)39 838 9492**
Norway (Oslo)	**47 23 12 06 05**	**47 23 12 05 75**
Poland, Lithuania, Latvia, and Estonia (Warszawa)	**48 (22) 866 40 10**	**48 (22) 866 40 20**
Portugal (Lisboa)	**351 (0)22 605 33 14**	**351 (0)22 605 33 15**
Russia (Moskva)	**7 502 935 8888**	**7 502 564 8787**
South East Europe (Zagreb, Croatia)	**385 1 34 91 927/838**	**385 1 34 91 840**
Spain (Tres Cantos)	**34 (0)91 806 1210**	**34 (0)91 806 1206**
Sweden (Stockholm)	**46 (0)8 619 4400**	**46 (0)8 619 4401**
Switzerland (Rotkreuz)	**41 (0)41 799 7777**	**41 (0)41 790 0676**

Region	Telephone Dial...	Fax Dial...
The Netherlands (Nieuwerkerk a/d IJssel)	**31 (0)180 392400**	**31 (0)180 392409** or **31 (0)180 392499**
United Kingdom (Warrington, Cheshire)	**44 (0)1925 825650**	**44 (0)1925 282502**
All other countries not listed (Warrington, UK)	**44 (0)1925 282481**	**44 (0)1925 282509**
Japan		
Japan (Hacchobori, Chuo-Ku, Tokyo)	**81 20 477392** (Toll free) or **81 3 5566 6230**	**81 20 477120** (Toll free) or **81 3 5566 6507**
Latin America		
Caribbean countries, Mexico, and Central America	**52 55 35 3610**	**52 55 66 2308**
Brazil	**0 800 704 9004** or **55 11 5070 9654**	**55 11 5070 9694/95**
Argentina	**800 666 0096**	**55 11 5070 9694/95**
Chile	**1230 020 9102**	**55 11 5070 9694/95**
Uruguay	**0004 055 654**	**55 11 5070 9694/95**

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We strongly encourage you to visit our Web site for answers to frequently asked questions and for more information about our products. You can also order technical documents or an index of available documents and have them faxed or e-mailed to you through our site. The Applied Biosystems Web site address is

http://www.appliedbiosystems.com/techsupp

To submit technical questions from North America or Europe:

Step	Action
1	Access the Applied Biosystems Technical Support Web site.
2	Under the **Troubleshooting** heading, click **Support Request Forms**, then select the relevant support region for the product area of interest.
3	In the **Personal Assistance** form, enter the requested information and your question, then click **Ask Us RIGHT NOW**.
4	In the **Customer Information** form, enter the requested information and your question, then click **Ask Us RIGHT NOW**. Within 24 to 48 hours, you will receive an e-mail reply to your question from an Applied Biosystems technical expert.

To Obtain Documents on Demand

Free, 24-hour access to Applied Biosystems technical documents, including MSDSs, is available by fax or e-mail or by download from our Web site.

To order documents...	Then...
by index number	a. Access the Applied Biosystems Technical Support Web site at **http://www.appliedbiosystems.com/techsupp** b. Click the **Index** link for the document type you want, then find the document you want and record the index number. c. Use the index number when requesting documents following the procedures below.
by phone for fax delivery	a. From the U.S. or Canada, call **1-800-487-6809,** or from outside the U.S. and Canada, call **1-858-712-0317**. b. Follow the voice instructions to order the documents you want. **Note** There is a limit of five documents per request.
through the Internet for fax or e-mail delivery	a. Access the Applied Biosystems Technical Support Web site at **http://www.appliedbiosystems.com/techsupp** b. Under **Resource Libraries**, click the type of document you want. c. Enter or select the requested information in the displayed form, then click **Search**. d. In the displayed search results, select a check box for the method of delivery for each document that matches your criteria, then click **Deliver Selected Documents Now** (or click the PDF icon for the document to download it immediately). e. Fill in the information form (if you have not previously done so), then click **Deliver Selected Documents Now** to submit your order. **Note** There is a limit of five documents per request for fax delivery but no limit on the number of documents you can order for e-mail delivery.

To Obtain Customer Training Information

The Applied Biosystems Training web site at **www.appliedbiosystems.com/techsupp/training.html** provides course descriptions, schedules, and other training-related information.

Troubleshooting B

Overview

About This Appendix

Troubleshooting information addresses possible causes of a number of certain observations. By following the recommended actions, these observations described in this appendix may be further understood and/or eliminated.

In This Appendix

Appendix B contains the following topics:

Topic	See Page
Overview	B-1
Troubleshooting	B-2

Observation	Possible Causes	Recommended Actions
Faint or no signal from both the AmpFℓSTR® Control DNA 9947A and the DNA test samples at all loci.	Incorrect volume or absence of either AmpFℓSTR® PCR Reaction Mix, AmpFℓSTR Identifiler™ Primer Set, or AmpliTaq Gold® DNA Polymerase.	Repeat amplification.
	No activation of AmpliTaq Gold DNA Polymerase.	Repeat amplification, making sure to hold reactions initially at 95 °C for 11 min.
	PCR Master Mix not vortexed thoroughly before aliquoting.	Vortex PCR Master Mix thoroughly.
	AmpFℓSTR Identifiler Primer Set exposed to too much light.	Store Primer Set protected from light.
	GeneAmp® PCR System malfunction.	Refer to the thermal cycler user's manual and check instrument calibration.
	Incorrect thermal cycler parameters.	Check the protocol for correct thermal cycler parameters.
	Tubes not seated tightly in the thermal cycler during amplification.	Push reaction tubes firmly into contact with block after first cycle. Repeat test.
	GeneAmp PCR System 9600 heated cover misaligned.	Align GeneAmp 9600 heated cover properly so that white stripes align after twisting the top portion clockwise.
	Wrong PCR reaction tube.	Use Applied Biosystems MicroAmp Reaction Tubes with Caps for the GeneAmp 9600 and 9700.
	MicroAmp® Base used with tray/retainer set and tubes in GeneAmp 9600 and 9700.	Remove MicroAmp Base from tray/retainer set and repeat test.
	Insufficient PCR product electrokinetically injected.	**For ABI PRISM® 310 runs:** Mix 1.5 µL of PCR product and 24.5 µL of Hi-Di™ Formamide/GeneScan™-500 LIZ™ solution.
	Degraded formamide.	Check the storage of formamide; do not thaw and re-freeze multiple times. Try Hi-Di™ Formamide.

Observation	Possible Causes	Recommended Actions
Positive signal from AmpFℓSTR Control DNA 9947A but no signal from DNA test samples	Quantity of test DNA sample is below assay sensitivity	Quantitate DNA and add 0.5–1.25 ng of DNA. Repeat test.
	Test sample contains PCR inhibitor (*e.g.*, heme compounds, certain dyes)	Quantitate DNA and add minimum necessary volume. Repeat test.
		Wash the sample in a Centricon®-100. Repeat test.
	Test sample DNA is degraded	If possible, evaluate the quality of DNA sample by running an agarose gel. If DNA is degraded, re-amplify with an increased amount of DNA.
	Dilution of test sample DNA in H_2O or wrong buffer (*e.g.*, wrong EDTA concentration)	Re-dilute DNA using TE Buffer (with 0.1-mM EDTA).
More than two alleles present at a locus	Presence of exogenous DNA	Use appropriate techniques to avoid introducing foreign DNA during laboratory handling.
	Too much DNA in reaction	Use recommended amount of template DNA (0.5–1.25 ng).
	Mixed sample	See Chapter 4, “Experiments and Results.”
	Amplification of stutter product (n-4 bp position)	
	Incomplete 3´ A base addition (n-1 bp position)	See Chapter 4, “Experiments and Results.” Be sure to include the final extension step of 60 °C for 60 min in the PCR.
	Signal exceeds dynamic range of instrument (off-scale data).	Quantitate DNA and re-amplify sample, adding 0.5–1.25 ng of DNA.
	Poor spectral separation (bad matrix).	Follow the steps for creating a matrix file.
		Confirm that Filter Set G5 modules are installed and used for analysis.
Some but not all loci visible on electropherogram	Test sample DNA is degraded	If possible, evaluate the quality of DNA sample by running an agarose gel. If DNA is degraded, re-amplify with an increased amount of DNA.
	Test sample contains PCR inhibitor (*e.g.*, heme compounds, certain dyes)	Quantitate DNA and add minimum necessary volume. Repeat test.
		Wash the sample in a Centricon-100. Repeat test.

Observation	Possible Causes	Recommended Actions
ABI PRISM 310 Genetic Analyzer		
Data was not automatically analyzed	Sample sheet not completed	Complete sample sheet as described.
	Injection list not completed	Complete injection list as described.
	Preferences not set correctly in ABI PRISM® 310 Data Collection Software	In **Preferences** under the **Window** menu, select **Injection List Defaults** and the **Autoanalyze** checkbox.
Extra peaks visible when sample is known to contain DNA from a single source	Incomplete denaturation before loading onto detection instrument	Heat samples to 95 °C for 3 min in deionized formamide solution. Snap cool on ice. Use Genetic Analyzer 0.5-mL Sample Tubes and a thermal cycler.
Current too high	Decomposition of urea in the POP-4™ polymer solution	Add fresh POP-4 polymer solution to the syringe.
	Incorrect buffer concentration	Replace buffer with 1X Genetic Analyzer Buffer.
No current	No 1X Genetic Analyzer buffer	Refill buffer vials with 1X Genetic Analyzer buffer.
	Pump block channel blockage	Remove and clean block. Refer to the ABI PRISM 310 *Genetic Analyzer User's Manual.*
	Loose valve fittings or syringe	Tighten valve fittings and syringe.
	Capillary not flush with electrode	Tape capillary securely to heat plate. Refer to the *ABI PRISM 310 Genetic Analyzer User's Manual* (P/N 903565).
	Electrode bent	Check calibration of autosampler.
No signal	Capillary misaligned with electrode	Align capillary and electrode.
	No PCR product added	Add 1.5-µL PCR product to formamide/GeneScan-500 LIZ mixture.
	Capillary bent out of sample tube	Align capillary and electrode. Recalibrate autosampler.
	Autosampler not calibrated correctly	Calibrate autosampler in X, Y, and Z directions.
	PCR product not at bottom of tube	Spin sample tube in microcentrifuge.
	Air bubble at bottom of sample tube	Spin tube in microcentrifuge to remove air bubbles.
	Sealed sample tube septum	Replace septum.

Observation	Possible Causes	Recommended Actions
Low signal	PCR product added to non-deionized formamide	Always use deionized formamide for sample preparation. Verify conductivity is < 30-μ siemens.
	PCR product not mixed well with formamide/GeneScan-500 LIZ mixture	Mix PCR product with formamide/GeneScan-500 LIZ mixture by pipetting up and down several times.
Loss of resolution after 100 bp	Excess salt in sample	Do not concentrate PCR product by evaporation. Use Centricon-100 if necessary.
	Too much DNA in sample	Treat and dilute the PCR product.
	Bad water	Use autoclaved or freshly prepared deionized water.
	Incorrectly prepared and/or old solutions	Replace buffer and polymer with fresh solutions.
Runs get progressively slower, *i.e.*, size standard peaks come off at higher and higher scan numbers	Leaking syringe: polymer is not filling capillary before every injection	Clean syringe thoroughly.
		Replace syringe.
Runs get progressively faster, *i.e.*, size standard peaks come off at lower and lower scan numbers	Water in syringe	Prime syringe with small volume of polymer and discard. Fill syringe with polymer.
High baseline	Dirty capillary window	Clean capillary window with 95% ethanol.
	Capillary moved out of position in laser window.	Position capillary in front of laser window.
	Cracked capillary	Replace the capillary

C

Laboratory Setup

Overview

About This Appendix

This appendix provides some references for laboratories preparing to implement PCR technology. Careful planning and design of the laboratory, and training of all laboratory personnel, are necessary to ensure that exogenous DNA and PCR products are confined to designated areas.

In This Appendix

Appendix C contains the following topics:

Topic	See Page
Overview	C-1
Lab Design	C-2

Lab Design

Sensitivity of PCR

Many resources are available for the appropriate design of a PCR laboratory. If you are using the Identifiler kit for forensic DNA testing, you may want to refer to http://www.ojp.usdoj.gov/nij/scidocs.htm, "Forensic Laboratories: Handbook for Facility Planning, Design, Construction and Moving." If you are using the Identifiler kit for parentage DNA testing, you may want to refer to the "Standards for Parentage Testing Laboratories." The sensitivity of the AmpFℓSTR® Identifiler™ PCR Amplification Kit (and other PCR-based tests) permits amplification of minute quantities of DNA. This necessitates precautions to avoid contamination of samples yet to be amplified (Kwok and Higuchi, 1989).

While contamination of amplified DNA with unamplified DNA (genomic DNA) does not pose a problem, ordinary precautions, such as changing pipet tips between samples, should be taken when handling and analyzing PCR product. These precautions should prevent cross-contamination between samples of amplified DNA.

Care should be taken while handling and processing samples to prevent chance contamination by human DNA. Gloves should be worn at all times and changed frequently. Sample tubes should be closed when not in use. Dispersal of aerosols should be limited through careful handling of sample tubes and reagents.

Applied Biosystems does not intend these references for laboratory design to constitute all precautions and care necessary using PCR technology.

Extra precautions and care should be taken during DNA extraction and PCR setup to prevent transfer of DNA from one sample to another. Use a new, filter-plugged pipet tip for each sample, open tubes carefully, and keep sample tubes closed when not in use. Applied Biosystems does not intend these references for laboratory design to constitute all precautions and care necessary when using PCR technology.

DNA Extraction Protocols

D

Overview

About This Appendix

Appendix D describes some extraction methods for various DNA samples.

In This Appendix

Appendix D contains the following topics:

Topic	See Page
Overview	D-1
Introduction	D-2
Collection and Storage of Samples for DNA Extraction	D-3

Introduction

Overview of DNA Sample Types

Many DNA extraction procedures have been developed. Both manual and automated extraction procedures can be further divided into organic and non-organic procedures. Depending upon the material received, the scientist will need to determine which procedure is appropriate for each piece of evidence.

DNA for PCR amplification and analysis using the AmpFℓSTR® Identifiler™ PCR Amplilfication Kit may be extracted from fresh or frozen whole blood, peripheral blood lymphocytes, blood stains, sperm cells, paraffin blocks, teeth, hair, tissue, bone, and other biological samples.

DNA Extraction Methods

There are numerous procedures that are currently being used for DNA extraction. Some of these extraction procedures include Chelex®, phenol-chloroform and FTA™ paper. Regardless of the method used for DNA extraction, all samples must be handled carefully to prevent sample-to-sample contamination or contamination by extraneous DNA. Also, when possible, we recommend that the samples should be processed at a separate time from reference samples.

Phenol-Chloroform Method

The phenol-chloroform method removes proteins and other cellular components from nucleic acids, resulting in relatively purified DNA preparations. This method results in double-stranded DNA that is suitable for AmpFℓSTR Identifiler kit amplifications. DNA extracted by the phenol-chloroform method is also suitable for RFLP analysis provided it is not significantly degraded. This method is also recommended when extracting DNA from relatively large samples (*i.e.*, when the amount of DNA in a sample is expected to be greater than 100 ng).

Chelex Method

The Chelex method of DNA extraction is more rapid than the phenol-chloroform method. It involves fewer steps, resulting in fewer opportunities for sample-to-sample contamination. This method produces single-stranded DNA that is suitable for AmpFℓSTR Identifiler kit amplification. DNA extracted with Chelex cannot be used for RFLP analysis.

FTA Paper Extraction

The FTA paper extraction begins immediately when blood is spotted on FTA paper. The cells are lysed and the DNA is immobilized within the matrix of the paper. The DNA is purified by performing a series of washes, after which the DNA is ready for PCR amplification.

Warnings to Users

Read the Material Safety Data Sheet (MSDS) and label warning furnished by the supplier of each chemical or reagent for the correct handling and the proper use of protective equipment. For additional copies of an MSDS call 1-800-327-3002.

Collection and Storage of Samples for DNA Extraction

Proper Collection

Storage of various DNA specimens is an essential step to insuring that the DNA profiles obtained are accurate and meaningful. Proper chain of custody is vital to maintaining the integrity of each particular specimen.

DNA Quantitation

E

Overview

About This Appendix

This appendix discusses the importance of quantitating DNA samples prior to amplification. The QuantiBlot® Human DNA Quantitation Kit is described and can be used for the quantitation of samples.

In This Appendix

Appendix E contains the following topics:

Topic	See Page
Overview	E-1
Importance of Quantitation	E-2
Using the QuantiBlot Kit	E-3
Commonly Asked Questions about the QuantiBlot Kit	E-5

Importance of Quantitation

DNA Quality

The DNA quality (degree of degradation), purity, and total quantity in a sample influences the efficiency of a PCR amplification. Lack of amplification is usually due to highly degraded DNA, the presence of PCR inhibitors, insufficient DNA quantity, or any combination of these factors.

Quantitation and PCR Amplification

The QuantiBlot Human DNA Quantitation Kit (P/N N808-0114) is an ideal method for accurate quantitation of human DNA (Walsh *et al.*, 1992). If the QuantiBlot kit determines that sufficient DNA is present in the extracted sample (greater than approximately 0.05-ng/µL concentration), then lack of amplification is most likely due to PCR inhibitors or severe degradation of the DNA.

Quantitation of samples shows if there is a sufficient amount of DNA present for amplification. Also, PCR inhibition can be minimized by adding the smallest volume of DNA extract necessary for successful amplification (volume containing approximately 0.5–1.25 ng). Lastly, by using the minimal volume of extracted DNA for PCR, the number of different genetic marker tests or repeat analyses that can be performed is maximized. Likewise, informed decision(s) can be made regarding typing of samples present in extremely limiting quantities.

DNA quantitation is particularly important for amplifications using the AmpFℓSTR® Identifiler™ kit where optimal results are obtained using a range of 0.5–1.25 ng of input DNA. Adding greater than 1.25 ng of DNA can result in too much PCR product, such that the dynamic range of the instrument used to detect and analyze the PCR product is exceeded.

See “Off-Scale Data” on page 3-34 for more details.

Using the QuantiBlot Kit

How the Kit Works

The method of DNA quantitation using the QuantiBlot Human DNA Quantitation Kit is based on probe hybridization to the human alpha satellite locus, D17Z1. A biotinylated probe specific for the D17Z1 sequence is hybridized to sample DNA that has been immobilized via slot blot onto a nylon membrane.

The subsequent binding of horseradish peroxidase/streptavidin enzyme conjugate (HRP-SA) to the bound probe allows for either colorimetric or chemiluminescent detection. In the case of colorimetric detection, the oxidation of 3,3´,5,5´-tetramethylbenzidine (TMB) catalyzed by HRP-SA results in the formation of a blue precipitate directly on the nylon membrane.

For chemiluminescent detection, the oxidation of a luminol-based reagent catalyzed by HRP-SA results in the emission of photons that are detected on standard autoradiography film. This process is called enhanced chemiluminescence (ECL).

In both cases, the quantity of sample DNA is determined by comparison of the sample signal intensity to human DNA standards that have been calibrated against two DNA controls of known quantity.

The colorimetric method allows for detection and quantitation down to 150 pg. The chemiluminescent method can detect 150 pg with a 15-minute exposure to film and can detect as little as 20 pg with longer film exposures (3 hours to overnight). Results obtained from various biological samples using the QuantiBlot Kit are shown in Figure E-1 on page E-4.

Note For specific procedures, refer to the QuantiBlot Human DNA Quantitation Kit product insert.

Specificity for Primate DNA

One significant advantage offered by the QuantiBlot kit is that the probe is highly specific for human/primate DNA. When tested, 300-ng quantities of several non-primate DNA samples (*E. coli*, yeast, dog, cat, mouse, rat, pig, cow, chicken, fish, and turkey) were found to give either no signals or signals that were less than or equal to that obtained for 0.15 ng of human DNA. This high degree of specificity for human/primate DNA allows for the accurate quantitation of target human DNA in samples that also contain significant amounts of microbial or other non-primate DNA.

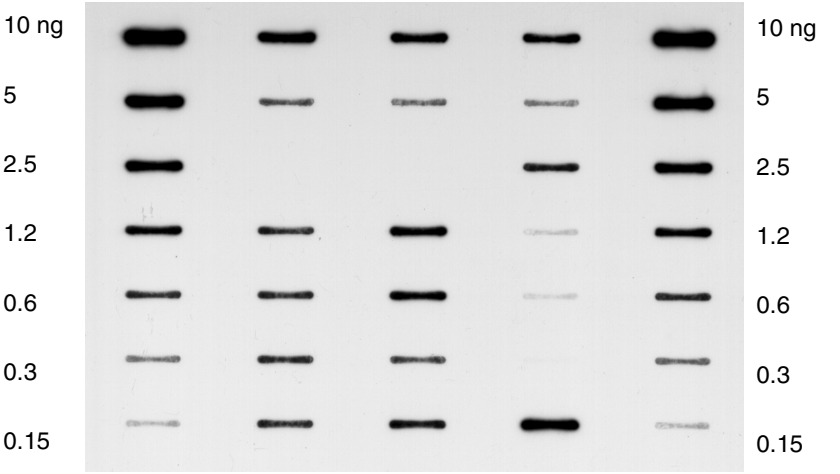

Figure E-1 QuantiBlot Human DNA Quantitation Kit results (ECL detection)

Single-Stranded and Degraded DNA

Another advantage of the QuantiBlot kit method is that single-stranded and/or non-purified DNA samples can be quantitated. DNA samples extracted using the Chelex method can be quantitated, as can those extracted by other methods, including phenol-chloroform, salting out, and binding to silica particles.

Degraded DNA gives the same results as fully intact DNA over a wide range of average DNA sizes. However, DNA quantity can be underestimated when the DNA is extremely degraded. For example, experimental results indicated that the signal obtained for DNA degraded to an average size of 500–2000 bp was about half of the expected intensity.

Extremely degraded DNA usually amplifies less efficiently than intact DNA, so a greater quantity of degraded DNA may be required to give the same results as intact DNA.

Commonly Asked Questions about the QuantiBlot Kit

How Much DNA?

How much of the DNA extract should be added to the amplification reaction if a sample gives no signal for the QuantiBlot assay?

As an example, assume that 5 µL of the DNA extract is spotted, and the lowest DNA standard tested is 150 pg. So, the DNA concentration in the sample must be less than 150 pg/5 µL or 30 pg/µL. The quantity of DNA in 10 µL of extract, which is the maximum that can be added to an AmpFℓSTR Identifiler kit amplification, would therefore be less than 0.3 ng.

The possible approaches that can be taken for such a sample include the following:

- Attempt amplification using 10 µL of the extract.
- Concentrate the sample to a smaller volume using a Centricon®-100 before amplification.

Multiple Film Exposures

Is it possible to perform multiple film exposures with the ECL detection method?

Yes. In fact, a wise strategy is to perform a 15-minute film exposure first, which gives sensitivity down to at least 150 pg. Then place the film on the membrane for 3 hours or as long as overnight. The longer exposure will give sensitivity down to about 20 pg.

The photon emission kinetics of ECL are such that many exposures can be taken in a relatively short period of time. The light output is the greatest in the first hour, gradually decreasing over the next several hours with a half-life of about 60 minutes. The results of one experiment, for example, indicated that six exposures could be taken in the first 2.5 hours of photon emission, with each exposure detecting 80–150 pg of DNA. A seventh exposure with the film on the membrane overnight was easily able to detect the 80 pg DNA sample.

Sometimes it is beneficial to perform a very short exposure (about 5 minutes) to facilitate quantitation of samples having intense signals in the range of 5–10 ng DNA.

Repeating the Assay

Can the probe be stripped off the membrane so that the QuantiBlot assay can be repeated if a mistake is made during the hybridization/detection steps?

Yes, for the ECL method. This procedure can be used with the TMB method only if no blue precipitate was deposited on the membrane.

The procedure is as follows:

Step	Action
1	Heat 150 mL of the Wash Solution (1.5X SSPE, 0.5% SDS) to approximately 90 °C in a glass bowl.
2	Take the Wash Solution off the heat source and place the nylon QuantiBlot membrane (containing the spotted samples) into the solution.
3	Rotate on an orbital shaker at room temperature for 20 min.
4	Remove the membrane from the Wash Solution. **IMPORTANT** Do not let the QuantiBlot membrane dry out at any time.
5	Begin the QuantiBlot kit protocol starting at the hybridization step (refer to the QuantiBlot Human DNA Quantitation Kit product insert).

Performing Hybridization and Detection at a Later Time

Is it possible to spot the samples onto the membrane and then perform the hybridization and detection steps at a later time?

Yes. Proceed as follows:

Step	Action
1	Immediately after spotting the samples onto the membrane, place the membrane in 100 mL of 5X SSPE (without SDS).
2	Store at 2–6 °C protected from light.
3	Resume the protocol beginning with the pre-hybridization step (Section 4.1 in the QuantiBlot Human DNA Quantitation Kit product insert). For best sensitivity, resume the protocol within 24 hr.

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F

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Index

A

A nucleotide
  addition by AmpliTaq Gold to 3´ end of amplicon 4-24
ABI PRISM 310 Genetic Analyzer
  protocol 3-3 to 3-37
    data analysis 3-26 to 3-29
    ending the run 3-34
    preparing and loading samples 3-22 to 3-23
    sample electrophoresis 3-24 to 3-25
    setting up a run 3-5 to 3-11
    setting up the instrument 3-4 to 3-5
  using Performance Optimized Polymer 4 for analysis 3-2 to 3-34
agarose gel, using to examine DNA 4-35
allele frequencies in the population databases 4-44 to 4-53
AmpFℓSTR Allelic Ladders
  calculating precision data using the allelic ladders 4-7 to 4-17
  using to determine genotypes 5-19 to 5-21
AmpFℓSTR Identifiler
  loci
    allele frequencies in the population databases 4-44 to 4-53
    Probability of Exclusion (P_E) from Paternity 4-56
    Probability of Identity (P_I) 4-55
AmpFℓSTR Identifiler Kit Template 5-3 to 5-16
  examining data 5-6 to 5-7
  making tables 5-7 to 5-9
  troubleshooting genotyping 5-10
  understanding the template kit 5-10 to 5-16
  using the kit 5-4 to 5-5
amplification
  differential amplification of loci 4-34
AmpliTaq Gold DNA Polymerase
  catalyzing the addition of a 3´ A nucleotide 4-24
automated genotyping
  about the software 5-2
  AmpFℓSTR Identifiler Kit Template
    examining data 5-6 to 5-7
    making tables 5-7 to 5-9
    troubleshooting genotyping 5-10
    understanding the template kit 5-10 to 5-16
    using the kit 5-4 to 5-5
  AmpFℓSTR Identifiler Kit Template 5-3 to 5-16
  before running Genotyper 5-2 to 5-3

C

contamination C-2
customer support. *See* technical support A-1

D

degraded DNA 4-34 to 4-35
differential amplification
  of loci 4-34
DNA
  amplification using bloodstained FTA cards 2-9
  effect of DNA quantity on results 2-7 to 2-9, 4-31 to 4-32
  extraction protocols D-2 to D-3
    collecting and storing samples D-3
  how degraded DNA affects which loci amplify 4-34 to 4-35
  mixed samples causing extra peaks 4-38 to 4-41
    detecting 4-38 to 4-39
    detection limit 4-40 to 4-41
    resolving 4-39 to 4-41
  quantitation E-2 to E-6
  using agarose gel analysis to examine the DNA 4-35
Documents on Demand A-6

E

effect of DNA quantity on results 2-7 to 2-9
effect of inhibitors 4-33 to 4-34
electropherogram

causes for extra peaks 4-40 to 4-41
addition of a 3´ A nucleotide 4-24
DNA from more than one individual 4-38 to 4-41
stutter peak 4-18 to 4-23
viewing the results 3-30 to 3-32
e-mail, address for technical support A-1
evidence
exclusion of suspects 4-43
extraction protocols D-2 to D-3
collecting and storing samples D-3

F

Five-Dye Analysis 1-2 to 1-5
FTA cards
using bloodstained FTA cards for amplification 2-9

G

gels
using an agarose gel to examine DNA 4-35
GeneScan Analysis software
viewing results 3-30
GeneScan-500 LIZ Internal Lane Size Standard 3-22, 3-30, 4-7, 5-19 to 5-20
genetics 4-43 to 4-52
allele frequencies 4-44 to 4-53
populations and samples used in studies 4-43
probability of identity 4-55
probability of paternity exclusion 4-56
genotype
determining 4-6
AmpFℓSTR Allelic Ladders 5-19 to 5-21
calculating precision data using the allelic ladders 4-7 to 4-17
exclusion of suspects 4-43
resolving in mixed samples 4-40 to 4-41
guidelines
laboratory setup C-1
validation studies 4-2 to 4-36

H

help. *See* technical support A-2
hematin, effect on DNA samples 4-33 to 4-34

I

Internet address
customer training information A-6
Documents on Demand A-6

L

laboratory setup C-1
loci
allele frequencies in the population databases 4-44 to 4-53
differential amplification 4-34
lack of amplification
effect of DNA quantity on results 4-31 to 4-32
population data 4-43 to 4-53
allele frequencies 4-44 to 4-53
probability of identity 4-55
probability of paternity exclusion 4-56
samples used in studies 4-43
locus. *See* loci

M

matrix file 3-12

P

PCR
amplification of tetranucleotide STR loci (stutter peak) 4-18 to 4-23
inhibitor
causing lack of amplification 4-33 to 4-34
minimizing E-2
troubleshooting B-1 to B-5
PCR product
precautions when handling the PCR product C-2
using too much PCR product E-2
See Also PCR
phenol-chloroform DNA extraction D-2
population
genetics 4-43 to 4-52
allele frequencies 4-44 to 4-53
populations and samples used in the studies 4-43
Probability of Exclusion (P_E) from Paternity 4-56

probability of identity 4-55
Probability of Identity (P_I) 4-55
probability of paternity exclusion 4-56
See Also allele
protocols
ABI PRISM 310 Genetic Analyzer 3-3 to 3-37
data analysis 3-26 to 3-29
ending the run 3-34
preparing and loading samples 3-22 to 3-23
sample electrophoresis 3-24 to 3-25
setting up a run 3-5 to 3-11
setting up the instrument 3-4 to 3-5
using Performance Optimized Polymer 4 for analysis 3-2 to 3-34
DNA extraction D-2 to D-3
collecting and storing samples D-3

Q

QuantiBlot Human DNA Quantitation Kit
commonly asked questions E-5 to E-6
using for quantitation of human DNA E-2
using the kit E-3 to E-4
quantitation E-2 to E-6
commonly asked questions E-5 to E-6
QuantiBlot analysis E-3 to E-4

R

results. *See* viewing results

S

samples
collecting and storing for extraction of DNA D-3
DNA from more than one individual 4-38 to 4-41
detecting 4-38 to 4-39
detection limit 4-40 to 4-41
resolving genotypes in mixed samples 4-40 to 4-41
setting up laboratories. *See* laboratory setup
STRBase 4-52
stutter peak or product 4-18 to 4-23

T

tables
allele frequencies of AmpFℓSTR Identifiler loci 4-44 to 4-53
precision results from the two instrument platforms 4-10 to 4-15
troubleshooting B-1 to B-5
technical support A-1 to A-6
e-mail address A-1
Internet address A-5
regional sales offices A-3 to A-5
telephone/fax (North America) A-2
training
obtaining information A-6
troubleshooting B-1 to B-5
automated genotyping 5-10

V

validation of the AmpFℓSTR Identifiler kit loci 4-2
kit reproducibility 4-6
minimum sample requirement 4-42
mixed specimen studies 4-42
mode of inheritance 4-27
optimizing PCR components 4-3
thermal cycler parameters 4-4
validation studies
general considerations 4-2 to 4-36
viewing results 3-31 to 3-32
GeneScan Analysis software 3-30
information provided in the electropherogram 3-30 to 3-32

W

WWW address
Applied Biosystems A-5
Documents on Demand A-6

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